KN

PCT WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) **Internationale Patentklassifikation** 7 :
A61K 38/37, 38/36, 38/48, A61P 7/04 // (A61K 38/37, 38:17, 38:57, 38:49, 38:40, 38:16)

A3

(11) **Internationale Veröffentlichungsnummer:** **WO 00/27425**

(43) **Internationales Veröffentlichungsdatum:** 18. Mai 2000 (18.05.00)

(21) **Internationales Aktenzeichen:** PCT/AT99/00271

(22) **Internationales Anmeldedatum:** 10. November 1999 (10.11.99)

(30) **Prioritätsdaten:**
A 1873/98 10. November 1998 (10.11.98) AT

(71) **Anmelder** *(für alle Bestimmungsstaaten ausser US)*: BAXTER AKTIENGESELLSCHAFT [AT/AT]; Industriestrasse 67, A–1221 Wien (AT).

(72) **Erfinder; und**
(75) **Erfinder/Anmelder** *(nur für US)*: SCHWARZ, Hans–Peter [AT/AT]; Schindlergasse 32, A–1180 Wien (AT). TURECEK, Peter [AT/AT]; Hauptstrasse 59g, Weidling, A–3400 Klosterneuburg (AT). BINDER, Bernd [AT/AT]; Freyung 6/6/9, A–1010 Wien (AT).

(74) **Anwälte:** SONN, Helmut usw.; Riemergasse 14, A–1010 Wien (AT).

(81) **Bestimmungsstaaten:** AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht
Mit internationalem Recherchenbericht.

(88) **Veröffentlichungsdatum des internationalen Recherchenrichts:** 31. August 2000 (31.08.00)

(54) **Title:** PHARMACEUTICAL PREPARATION CONTAINING A RECEPTOR–ANTAGONIST FOR TREATING BLOOD–CLOTTING DISORDERS

(54) **Bezeichnung:** PHARMAZEUTISCHE PRÄPARATION, ENTHALTEND EINEN REZEPTOR–ANTAGONISTEN ZUR BEHANDLUNG VON BLUTGERINNUNGSSTÖRUNGEN

(57) **Abstract**

The invention relates to a pharmaceutical preparation for treating blood–clotting disorders which is characterized in that it contains at least one protein selected from the group of pro–proteins of the blood clotting and additionally contains a coagulation–physiologically inert receptor binding competitor. The invention also relates to the medical application of said preparation.

(57) **Zusammenfassung**

Beschrieben wird eine pharmazeutische Präparation zur Behandlung von Blutgerinnungsstörungen, die dadurch gekennzeichnet ist, daß sie mindestens ein Protein, ausgesucht aus der Gruppe von pro–Proteinen der Blutgerinnung und weiters einen gerinnungsphysiologisch inerten Rezeptor–Bindungskompetitor enthält, sowie deren medizinische Verwendung.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AL	Albanien	**ES**	Spanien	**LS**	Lesotho	**SI**	Slowenien
AM	Armenien	**FI**	Finnland	**LT**	Litauen	**SK**	Slowakei
AT	Österreich	**FR**	Frankreich	**LU**	Luxemburg	**SN**	Senegal
AU	Australien	**GA**	Gabun	**LV**	Lettland	**SZ**	Swasiland
AZ	Aserbaidschan	**GB**	Vereinigtes Königreich	**MC**	Monaco	**TD**	Tschad
BA	Bosnien-Herzegowina	**GE**	Georgien	**MD**	Republik Moldau	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagaskar	**TJ**	Tadschikistan
BE	Belgien	**GN**	Guinea	**MK**	Die ehemalige jugoslawische Republik Mazedonien	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Griechenland			**TR**	Türkei
BG	Bulgarien	**HU**	Ungarn	**ML**	Mali	**TT**	Trinidad und Tobago
BJ	Benin	**IE**	Irland	**MN**	Mongolei	**UA**	Ukraine
BR	Brasilien	**IL**	Israel	**MR**	Mauretanien	**UG**	Uganda
BY	Belarus	**IS**	Island	**MW**	Malawi	**US**	Vereinigte Staaten von Amerika
CA	Kanada	**IT**	Italien	**MX**	Mexiko		
CF	Zentralafrikanische Republik	**JP**	Japan	**NE**	Niger	**UZ**	Usbekistan
CG	Kongo	**KE**	Kenia	**NL**	Niederlande	**VN**	Vietnam
CH	Schweiz	**KG**	Kirgisistan	**NO**	Norwegen	**YU**	Jugoslawien
CI	Côte d'Ivoire	**KP**	Demokratische Volksrepublik Korea	**NZ**	Neuseeland	**ZW**	Zimbabwe
CM	Kamerun			**PL**	Polen		
CN	China	**KR**	Republik Korea	**PT**	Portugal		
CU	Kuba	**KZ**	Kasachstan	**RO**	Rumänien		
CZ	Tschechische Republik	**LC**	St. Lucia	**RU**	Russische Föderation		
DE	Deutschland	**LI**	Liechtenstein	**SD**	Sudan		
DK	Dänemark	**LK**	Sri Lanka	**SE**	Schweden		
EE	Estland	**LR**	Liberia	**SG**	Singapur		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/AT 99/00271

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/37 A61K38/36 A61K38/48 A61P7/04 //(A61K38/37, 38:17,38:57,38:49,38:40,38:16)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 34930 A (EIBL JOHANN ;IMMUNO AG (AT); MITTERER ARTUR (AT); DORNER FRIEDRICH) 25 September 1997 (1997-09-25) abstract; claims 1,16,18 page 1, line 1 -page 3, line 11	1-5,9, 12,13, 15-17, 19,20, 22,23,25
X	EP 0 519 900 A (IMMUNO AG) 23 December 1992 (1992-12-23) abstract; claims 1-3 page 2, left-hand column, line 1 -right-hand column, line 41	1,2,4,5, 9,12,13, 15,17,20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 May 2000	13/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Niemann, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 32459 A (STRINGER KATHLEEN A ;REPINE JOHN E (US); MCCORD JOE M (US); HYBERT) 30 July 1998 (1998-07-30) page 3, line 28 - line 29 page 14, line 16 -page 15, line 2; claims 1,13	14
X	WO 95 25536 A (UNIV WASHINGTON ;JEWISH HOSPITAL OF ST LOUIS (US); SCHWARTZ ALAN L) 28 September 1995 (1995-09-28) page 2, line 20 -page 3, line 31; claims 1-3,7-9,11	23
X	WO 94 14471 A (UNIV WASHINGTON) 7 July 1994 (1994-07-07) abstract; claim 1 page 13, line 12 -page 14, line 23 page 15, line 1 - line 22	23
A	NEELS ET AL: "ligand receptor interaction of the low density lipoprotein receptor-related protein, a multi-ligand endocytic receptor" FIBRINOLYSIS & PROTEOLYSIS, vol. 12, no. 4, 1998, pages 219-240, XP000907114 abstract page 220, left-hand column, line 48 -page 222, right-hand column, line 50; table 1 page 227, left-hand column, line 1 -page 231, right-hand column, line 5	13
A	NOORMAN F ET AL: "regulation of tissue-type plasminogen activator concentration by clearance via the mannose receptor and other receptors" FIBRINOLYSIS & PROTEOLYSIS, vol. 11, no. 4, 1997, pages 173-186, XP000907181 abstract page 174, left-hand column, line 16 -right-hand column, line 54 page 175, left-hand column, line 43 -right-hand column, line 47 page 176; table 1 page 177, right-hand column, line 7 -page 178, left-hand column, line 17 page 180, left-hand column, line 13 -right-hand column, line 54	13

-/--

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Inter. nales Aktenzeichen
PCT/AT 99/00271

Im Recherchenbericht angeführtes Pat ntdokument		Datum der Veröffentlichung	Mitglied(r) der Patentfamilie		Datum der Veröffentlichung
WO 9734930	A	25-09-1997	AT	403764 B	25-05-1998
			AT	49496 A	15-10-1997
			CA	2248960 A	25-09-1997
			EP	0888386 A	07-01-1999
EP 0519900	A	23-12-1992	AT	402263 B	25-03-1997
			AT	124091 A	15-08-1996
			AU	656199 B	27-01-1995
			AU	1830492 A	24-12-1992
			CA	2071625 A	21-12-1992
			JP	5170665 A	09-07-1993
			US	5830467 A	03-11-1998
WO 9832459	A	30-07-1998	AU	6053698 A	18-08-1998
WO 9525536	A	28-09-1995	US	5650391 A	22-07-1997
			AU	2159495 A	09-10-1995
WO 9414471	A	07-07-1994	US	5474766 A	12-12-1995
			AU	5955494 A	19-07-1994

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	YAKYAEV ET ALL: "cellular uptake and degradation of the thrombin activated factor VIII fragmets" BLOOD, vol. 90, no. 10, suppl 1, - 1997 page 31a XP000907187 cited in the application the whole document	1-25
A	NARITA MASAAKI ET AL: "The low-density lipoprotein receptor-related protein (LRP) mediates clearance of coagulation factor Xa in vivo." BLOOD JAN. 15, 1998, vol. 91, no. 2, 15 January 1998 (1998-01-15), pages 555-560, XP002137878 ISSN: 0006-4971 the whole document	1-25
A	KOUNNAS MARIA Z ET AL: "Cellular internalization and degradation of antithrombin III-thrombin, heparin cofactor II-thrombin, and alpha-1-antitrypsin-trypsin complexes is mediated by the low density lipoprotein receptor-related protein." JOURNAL OF BIOLOGICAL CHEMISTRY 1996, vol. 271, no. 11, 1996, pages 6523-6529, XP002137879 ISSN: 0021-9258 the whole document	1-25
P,X	SCHWARZ, HANS PETER ET AL: "Involvement of low-density lipoprotein receptor - related protein (LRP) in the clearance of factor VIII in von Willebrand factor -deficient mice" BLOOD (2000), 95(5), 1703-1708 , XP002137880 the whole document	1-7,9, 12,13, 15-17, 19,20, 22,23,25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AT 99/00271

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See supplemental sheet Additional Matter PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Continuation of Field I.2

Relevant Patent Claims Nos. 1-4, 7, 9, 12, 15-19 (Only Claim No. 8 can be dependent on Claim No. 7 since no reference to a mixture is given in Claims Nos. 1-6. Therefore, the search was directed at all of Claim No. 8.) relate to a compound which is respectively characterized by a desired feature, namely "receptor binding competitor".
(Only Claim No. 8 can be dependent on Claim No. 7 since no reference to a mixture is given in Claims Nos. 1-6. Therefore, the search was directed at all of Claim No. 8.)

The patent claims thus comprise all products, etc. which comprise this feature, whereas only a limited number of such products, etc. are supported by the description as per the patent application under the terms of PCT Article 5. In the case in question, the patent claims lack the corresponding support and the patent application lacks the necessary disclosure to such a degree that a meaningful search appears to be impossible to conduct with respect to the entire scope for which protection is sought.

For this reason, the search was directed at the sections of the patent claims which can be regarded as clear, supported or disclosed in the above-mentioned sense, namely to the sections relating to the LRP or mannose receptor binding competitor.

The applicant is therefore advised that patent claims or sections of patent claims laid to inventions for which no international search report was drafted normally cannot be the subject of an international preliminary examination (PCT Rule 66.1(e)). Similar to the authority entrusted with the task of carrying out the international preliminary examination, the EPO also does not generally carry out a preliminary examination of subject matter for which no search has been conducted. This is also valid in the case when the patent claims have been amended after receipt of the international search report (PCT Article 19), or in the case when the applicant submits new patent claims pursuant to the procedure in accordance with PCT Chapter II.

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/AT 99/00271

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 2 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. ☐ Ansprüche Nr.
weil sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

2. ☒ Ansprüche Nr. –
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich
siehe Zusatzblatt WEITERE ANGABEN PCT/ISA/210

3. ☐ Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 3 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. ☐ Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche.

2. ☐ Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Behörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. ☐ Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.

4. ☐ Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

Bemerkungen hinsichtlich eines Widerspruch ☐ Di zusätzlichen G bühren wurden vom Anmelder unter Widerspruch gezahlt.

☐ Die Zahlung zusätzlicher Recherchengebühren erfolgte ohn Widerspruch.

WEITERE ANGABEN PCT/ISA/ 210

Fortsetzung von Feld I.2

Die geltenden Patentansprüche 1-4, 7, 9, 12, 15-19 (Da es keine Referenz zu einem gemisch in den Ansprüchen 1-6 gibt, kann nur Anspruch 8 vom Anspruch 7 abhängig sein. Deswegen wurde die Recherche auf den ganzen Anspruch 8 gerichtet) beziehen sich auf eine Verbindung, jeweils charakterisiert durch eine erstrebenswerte Eigenschaft, nämlich "Rezeptor-Bindungskompetitor".
(Da es keine Referenz zu einem gemisch in den Ansprüchen 1-6 gibt, kann nur Anspruch 8 vom Anspruch 7 abhängig sein. Deswegen wurde die Recherche von dem ganzen Anspruch 8 gerichtet.)

Die Patentansprüche umfassen daher alle Produkte etc., die diese Eigenschaft aufweisen, wohingegen die Patentanmeldung Stütze durch die Beschreibung im Sinne von Art. 5 PCT nur für eine begrenzte Zahl solcher Produkte etc. liefert. Im vorliegenden Fall fehlen den Patentansprüchen die entsprechende Stütze bzw. der Patentanmeldung die nötige Offenbarung in einem solchen Maße, daß eine sinnvolle Recherche über den gesamten erstrebten Schutzbereich unmöglich erscheint.

Daher wurde die Recherche auf die Teile der Patentansprüche gerichtet, welche im o.a. Sinne als klar, gestützt oder offenbart erscheinen, nämlich die Teile betreffend die LRP oder Mannose-rezeptor Bindungkompetitor.

Der Anmelder wird darauf hingewiesen, daß Patentansprüche, oder Teile von Patentansprüchen, auf Erfindungen, für die kein internationaler Recherchenbericht erstellt wurde, normalerweise nicht Gegenstand einer internationalen vorläufigen Prüfung sein können (Regel 66.1(e) PCT). In seiner Eigenschaft als mit der internationalen vorläufigen Prüfung beauftragte Behörde wird das EPA also in der Regel keine vorläufige Prüfung für Gegenstände durchführen, zu denen keine Recherche vorliegt. Dies gilt auch für den Fall, daß die Patentansprüche nach Erhalt des internationalen Recherchenberichtes geändert wurden (Art. 19 PCT), oder für den Fall, daß der Anmelder im Zuge des Verfahrens gemäß Kapitel II PCT neue Patentanprüche vorlegt.

Pharmazeutische Präparation enthaltend einen Rezeptor-Antagonisten zur Behandlung von Blutgerinnungsstörungen

Die Erfindung betrifft eine pharmazeutische Präparation auf Basis von Blutgerinnungsproteinen und Maßnahmen zur Verlängerung deren biologischen Halbwertszeit.

Therapeutische Proteine, insbesondere Präparationen zur Behandlung von Blutgerinnungsstörungen, welche Blutgerinnungsproteine, wie z.B. von Willebrand-Faktor (vWF), beinhalten, haben oft relativ kurze Halbwertszeiten im Organismus. Dadurch kann eine therapeutische Verabreichung solcher Präparationen innerhalb kurzer Zeit wirkungslos werden oder zumindest in ihrer Wirksamkeit stark beeinträchtigt werden.

Vieles an den physiologischen Abbauwegen von Blutgerinnungsproteinen ist ebenso unbekannt wie die genauen Faktoren, die deren Halbwertszeit im Körper bestimmen.

So ist lediglich bekannt, daß aktivierter Faktor VIII über seine A2-Domäne an das Lipoproteinrezeptor-verwandte Protein (lipoprotein receptor related protein; LRP) bindet, wobei diese Bindung sowohl für die Internalisierung von Faktor VIII als auch für dessen Abbau zuständig ist. Es ist auch bekannt, daß durch ein Rezeptor-assoziiertes Protein (RAP), einem Inhibitor von LRP, die Internalisierung der A2-Domäne von Faktor VIII gehemmt werden kann, wobei angenommen wird, daß die Dissoziation der A2-Untereinheit vom übrigen aktivierten Faktor VIII-Molekül reversibel ist.

Das "low density lipoprotein related protein" ist ein multifunktionell endozytotisch aktiver Rezeptor, der strukturell wie funktionell unterschiedliche Liganden bindet und endozytieren kann. RAP ("receptor-associated protein") inhibiert alle Interaktionen der Liganden mit LRP in vitro.

Prinzipiell wird vermutet, daß dieser Mechanismus von Bedeutung bei der Aktivitätsregulierung von Faktor VIIIa sein könnte (Blood 90 (10) Suppl. 1: 31a (1997)).

Es ist auch bekannt, daß LRP als Abbaurezeptor (Clearence receptor) für Gewebeplasminogenaktivator (tPA) fungieren kann, d.h. für dessen Entfernung aus der Blutzirkulation verantwortlich ist. Weiters scheinen dabei auch weitere Rezeptoren, wie z.B. der Mannose-Rezeptor, von Bedeutung zu sein. Andererseits ist auch bekannt, daß LRP bei der Clearence von vielen verschiedenen Liganden, wie Proteinasen, Inhibitoren, deren Komplexe mit Proteinasen, sowie verschiedenen Lipoproteinen, beteiligt sein kann. LRP kann sowohl zellgebunden vorliegen als auch als lösliche Form (Quinn K.A. et al., XVIIth Congress of the International Society of Thrombosis and Haemostasis, 1999, Abstract).

Es wurde gefunden, daß tPA eine sehr schnelle Clearence im Blutkreislauf erfährt, und daß Mutanten von tPA sowie Inhibitoren der Clearence dazu verwendet werden können, um die Dosierung von tPA bei der Thrombolysetherapie zu verringern (Fibrinolysis and Proteolysis (1997), S. 173-186).

In RAP-defizienten Mäusen konnte nachgewiesen werden, daß die Clearence von α_2-Makroglobulin in der Leber gestört ist. Hierbei wurde auch nachgewiesen, daß die Menge von reifem prozessierten LRP sowohl in der Leber als auch im Gehirn reduziert ist (PNAS, 92 (1995), S. 4537-4541).

In der EP-0 713 881-A2 werden von Willebrand-Faktor (vWF)-Konzentrate in Kombination mit Antithrombolytika und/oder Fibrinolytika beschrieben. Zweck dieser Präparate ist die Verringerung der Blutungsrisiken bei einer Antikoagulationstherapie.

In der US 5 304 383 wird eine pharmazeutische Präparation, enthaltend lys-Plasminogen in Kombination mit einem Serinproteaseinhibitor, wie Aprotinin oder α_2-Makroglobulin, beschrieben, welche zur Behandlung von Plasminogen-Mangel und Thrombosen eingesetzt werden kann.

Die Erfindung stellt sich zur Aufgabe, eine pharmazeutische Präparation zur Verfügung zu stellen, auf Basis von einem Blutgerinnungsprotein, welches sich durch eine verbesserte in vivo-Halbwertszeit auszeichnet. Die Erfindung stellt sich weiters zur

Aufgabe, die in vivo Halbwertszeit eines Blutgerinnungsproteins zu verlängern, um einerseits den endogenen Gehalt an dem Blutgerinnungsprotein zu stabilisieren und andererseits die Effizienz von einem exogenen Blutgerinnungsprotein zu steigern.

Die Aufgabe wird erfindungsgemäß durch eine pharmazeutische Präparation zur Behandlung von Blutgerinnungsstörungen gelöst, welche Präparation mindestens ein Protein, ausgesucht aus der Gruppe von einem pro-Protein der Blutgerinnung enthält. Diese pharmazeutische Präparation ist weiters durch einen Gehalt an einem Rezeptor-Bindungskompetitor gekennzeichnet, welcher alleine gerinnungsphysiologisch inert ist, wie beispielsweise Lipoproteine. Dadurch wird die Wirkung des Proteins auf die Blutgerinnung nicht unmittelbar beeinflußt, der Rezeptor-Bindungskompetitor trägt lediglich zur Stabilisierung des Proteins in vivo bei.

Das Protein in der erfindungsgemäßen Präparation ist vorzugsweise ein Blutgerinnungsprotein, ausgesucht aus der Gruppe von Faktor II, V, VII, VIII, IX, X, XI, XII, vWF und Protein C. Diese Proteine sind die Proformen von der gerinnungsphysiologisch wirksamen aktiven Form. Beispielsweise ist Faktor II (Prothrombin) die Proform des gerinnungsaktiven Thrombins, welches Fibrinogen zu Fibrin enzymatisch spaltet und damit ein Blutgerinnsel hervorruft. Zu diesen Proteinen zählen einerseits die Vitamin K-abhängigen Proteine, welche auch in einem Prothrombinkomplex-Präparat auf Basis der Faktoren II, IX und X und gegebenenfalls Faktor VII und Protein C vorkommen. Weiters ist ein mögliches Protein in der erfindungsgemäßen Präparation des vWF bzw. dessen analoge Proteine. vWF zeigt vor allem in Plättchenreichem Plasma seine Eigenständigkeit als Blutgerinnungsfaktor, unabhängig von seiner Rolle als Träger und Stabilisator des Faktor VIII (Beguin et al., Thrombosis and Haemostasis 78 (1), 590-594 (1997)). vWF wird beispielsweise aus Plasma oder einer Plasmafraktion gewonnen, kann aber auch durch rekombinante DNA-Technologie, durch Expression einer transformierten Zelle, hergestellt werden. Beispielsweise wird ein rekombinanter vWF nach der Vorschrift von Pannekoek (EP 0 197 592) bzw. Fischer et al. (WO 96/10584) hergestellt.

Auch die Gruppe der Blutgerinnungszymogene bzw. Vorstufen der gerinnungsphysiologisch aktiven Blutgerinnungsproteine sind einerseits aus Plasma oder einer Plasmafraktion erhältlich und entsprechen den nativen Proteinen. Andererseits können die nativen Proteine bzw. deren Derivate auch aus Zellkulturüberständen gewonnen werden. Die Zellen werden dabei vorzugsweise durch rekombinante DNA-Technologie transformiert, um die rekombinanten Formen der Blutgerinnungsproteine zu erhalten.

Mutanten bzw. Analoge sind insoweit in der erfindungsgemäßen Präparation enthalten, wie diese noch zur Behandlung von Blutgerinnungsstörungen eingesetzt werden können und an einen Rezeptor funktionell, also kompetitiv, binden können.

Als Rezeptor für die Proteine der erfindungsgemäßen Präparation ist vor allem der Lipoproteinrezeptor zu nennen bzw. LRP. Ein weiterer Rezeptor, an dem die Proteine binden, ist beispielsweise der Mannose-Rezeptor. Es hat sich erfindungsgemäß überraschenderweise herausgestellt, daß die Liganden des LRP vor allem für pro-Proteine der Blutgerinnung eine positive Wirkung in vivo zeigen, indem diese Liganden an den Rezeptor kompetitiv binden. Dies war insofern überraschend, da nicht vermutet werden konnte, daß pro-Proteine der Blutgerinnung und vWF an LRP binden können. LRP war vor allem zur Bindung von Enzymen bzw. deren Komplexe mit Inhibitoren bekannt. Durch die kompetitive Eigenschaft der Bindung an LRP wird die vorzeitige Bindung des Proteins in der pharmazeutischen Präparation bzw. des endogenen Blutgerinnungszymogens und vWF verhindert, wodurch dessen biologische Verfügbarkeit wesentlich verbessert wird.

Als Rezeptor-Bindungskompetitor können eine Reihe von Proteinen eingesetzt werden, wobei diejenigen bevorzugt sind, deren physiologische Toleranz geprüft ist.

Erfindungsgemäß bevorzugt ist der Rezeptor-Bindungskompetitor ausgewählt aus der Gruppe der Enzyme, insbesondere Tissue-type Plasminogenaktivator (tPA), Urokinase (u-PA), pro-Urokinase (pro-u-PA), Lipoproteinlipase (LPL) und Kallikrein; Inhibitoren, insbesondere Plasminogen-Aktivator-Inhibitor-1 (PAI-1), Tissue factor pathway Inhibitor (TFPI) und Aprotinin; Enzym-Inhibitor-

Komplexe, insbesondere t-PA-PAI-1, u-PA-PAI-1 und Thrombin-PAI-1, α_2-Macroglobulin-Proteinase (fast form) α_2M, α_2M^*, Pregnancy zone protein-Proteinase, Elastase-α^1-Antitrypsin, Thrombin-Antithrombin III, Thrombin-Heparin-Cofactor II und u-PA-Protease-Nexin I; Lipoproteine, insbesondere low density-Lipoprotein (LDL), Apolipoprotein E angereichertes β-very low density-Lipoprotein, (apo E-β-VLDL), LPL angereichertes VLDL (LPL-VLDL) und LPL angereichertes β-VLDL (LPL-β-VLDL); Matrix-Proteine, insbesondere Thrombospondin 1 und 2; Toxine und Viren, insbesondere Pseudomonas Exotoxin A, und Minor-group human Rhinovirus; oder andere Liganden, insbesondere Apolipoprotein E (apo E), Lactoferrin, Rezeptor-assoziiertes Protein (RAP), FVIII und vWF.

Der kompetitiv wirkende Ligand ist vorzugsweise ausgewählt unter den physiologischen Proteinen, die vorzugsweise in der Zirkulation als (humanphysiologische) extrazelluläre Proteine auftreten. Diese können in nativer Form eingesetzt werden, oder als deren Analoge mit der Eigenschaft der Bindung an den Rezeptor.

Bevorzugterweise handelt es sich bei den kompetitiv wirkenden Liganden um RAP oder ein Derivat davon. Besonders bevorzugt ist ein Fragment von RAP, das jene Domäne umfaßt, die die Bindung von Proteinen an LRP verhindert und dadurch die Internalisierung und Clearance der Proteine inhibiert (I. Warshawsky et al., J. Biol.Chem., 1993, vol. 15, pp. 22046-22054; Bu G., EMBO J., 1995, vol. 14, pp. 2269-2280). Es kann sich dabei um RAP oder ein Derivat davon handeln, das humanen Ursprungs ist, aber auch um tierisches RAP oder ein Derivat davon, beispielsweise aus Mäusen, Ratten oder anderen Tieren.

Um die Wirkung der pharmazeutischen Präparation bzw. der endogenen Proteine nicht zu beeinträchtigen, ist der Rezeptor-Bindungskompetitor ausgewählt unter den gerinnungsphysiologisch inerten Proteinen, darunter beispielsweise "low density lipoprotein" (LDL) und Apolipoprotein.

Als gerinnungsphysiologisch inerter Rezeptor-Bindungskompetitor können auch Gemische dieser Rezeptor-Bindungskompetitoren eingesetzt werden, die eventuell nicht als einzelne Proteine, jedenfalls aber im Gemisch keinen unerwünschten Einfluß auf das Ge-

rinnungssystem ausüben. Beispiele für solche Gemische sind Thrombin mit ATIII oder mit Heparin-Cofaktor II, oder mit Plasminogen-Aktivator-Inhibitor-1 (PAI-1), weiters auch Elastase und α_1 -Antitrypsin, tPA mit PAI-1. Beispielsweise ist ein bevorzugtes Gemisch tPA und Aprotinin, welches sich hervorragend zur Stabilisierung etwa des Faktor VIII in vivo eignet. Der gewählte Rezeptor-Bindungskompetitor ist hier also ein Ligand, dessen gerinnungsphysiologische Wirkung unterbunden ist, gegebenenfalls durch die Antagonisierung mit dem entsprechenden Inhibitor.

Anstelle eines Gemisches aus einem prinzipiell zwar wirksamen Rezeptor-Bindungskompetitor und einem geeigneten Antagonisten kann auch eine mutierte Form des Rezeptor-Bindungskompetitors verwendet werden, der durch geeignete Mutationen (z.B. im aktiven Zentrum) inaktiviert ist (dessen Affinität gegenüber dem Rezeptor aber nicht wesentlich verändert (verringert) ist.) Beispiele hierfür sind u.a. mutierte Formen von tPA, Urokinase, Kallikrein, Renin, Thrombin (WO 96/41868) etc.

Es war überraschend, daß rekombinanter tPA (rtPA) den endogenen Faktor VIII-Gehalt in einem vWF-defizienten Hund über einen längeren Zeitraum stabilisierte, wodurch die Hämophilie behandelt werden konnte. Obwohl der natürliche Stabilisator des Faktor VIII, nämlich vWF, fehlte, konnte der LRP-Ligand bzw. das Gemisch der LRP-Liganden die Funktion des vWF übernehmen. Der Faktor VIII-Gehalt wurde um ca. 50 % erhöht und blieb bei diesem Niveau mehrere Tage. Das Enzym tPA ist ein bekannter Ligand des LRP. Die Verabreichung des rtPA an ein Säugetier blockiert also LRP und verhindert somit den metabolischen Abbau der Gerinnungsfaktoren, wie z.B. Faktor VIII. Dieser blockierende Effekt wird noch durch Aprotinin verstärkt. Der Effekt von Aprotinin alleine konnte auch in einem vWF-defizienten Hund gezeigt werden, der eine Kombination von rvWF und Aprotinin erhielt. Dadurch wurde wiederum eine Erhöhung des Faktor VIII-Gehaltes über einen längeren Zeitraum bewirkt.

Ein weiterer bevorzugter Rezeptor-Bindungskompetitor ist Aprotinin, welches sich hervorragend zur Stabilisierung des vWF eignet. Aprotinin kann beispielsweise nicht nur in einer vWF-Präpa-

ration zur verbesserten biologischen Verfügbarkeit beitragen, sondern auch, als einzelne Wirksubstanz verabreicht, zu verbesserten Zustand eines vWF-defizienten Patienten beitragen.

In vielen Fällen wird ein Patient mit einem Mangel an einem bestimmten Protein dann mit dem jeweiligen Rezeptor-Bindungskompetitor behandelt, um die Bildung des endogenen Proteins zu ermöglichen bzw. das Protein zu stabilisieren. Dies ist bei Patienten mit einem phänotypischen Gerinnungsproteinmangel möglich.

Das erfindungsgemäße Präparat bzw. die genannten Wirksubstanzen werden vorzugsweise als behandelte Präparate bzw. Proteine eingesetzt, um eine Übertragung von möglicherweise vorhandenen Pathogenen, wie Viren oder Prionen (TBE), auszuschließen. Bei der Gewinnung aus biologischen Materialien, wie Blut, Plasma, Plasmafraktionen oder Zellkulturen, existiert ein Risiko der Kontamination mit Humanpathogenen, das jedoch durch eine entsprechende Behandlung zur Inaktivierung bzw. Abreicherung eliminiert werden kann. Zu den effektiven Maßnahmen zur Inaktivierung von Viren zählen beispielsweise die Behandlung mit organischen Lösungsmitteln und/oder Detergenzien (EP-0 131 740, EP-0 050 061, virus inactivating eluent), die Behandlung mit chaotropen Mitteln (WO 90 15 613) Hitzebehandlungsverfahren, vorzugsweise in lyophilisiertem, trockenem oder feuchtem Zustand (siehe EP-0 159 311), Kombinationsverfahren wie das der (EP-0 519901) und physikalische Methoden. Letztere bewirken die Inaktivierung von Viren, beispielsweise durch Bestrahlung mit Licht, etwa in Gegenwart von Photosensibilisatoren (EP-0 471 794 und WO/AT 97/00068).

Zu dem Abreicherungsverfahren der Humanpathogene zählen insbesondere die Filtrationen unter Verwendung von Ultrafiltern, Tiefenfiltern oder Nanofiltern (A 341/98). Aber auch Fällungsschritte bzw. andere Proteinreinigungsmaßnahmen, wie die der Adsorption, tragen zur Abreicherung von möglicherweise vorhandenen Pathogenen bei.

Die erfindungsgemäße Präparation kann einerseits als fertige Formulierung, d.h. als Gemisch des Proteins mit dem Rezeptor-Bindungskompetitor zur Verfügung gestellt werden. Andererseits

ist es auch möglich, ein Set zur Verfügung zu stellen, welches A) das Protein und B) den Rezeptor-Bindungskompetitor enthält. Das Set hat den Vorteil, daß die Dosis der einzelnen Komponenten variabel ist und die Form der Administration den vorliegenden Verhältnissen angepaßt werden kann. Beispielsweise ist neben der iv-Verabreichung auch die intramuskuläre, subkutane oder orale Verabreichung der Wirkstoffe möglich. Die Wirksubstanzen können gemeinsam, also simultan, aber auch parallel oder konsekutiv dem Patienten verabreicht werden. Bei Verwendung eines Sets ist die erfindungsgemäße Präparation vorzugsweise in Fertigspritzen mit den einzelnen Komponenten vorgefertigt.

Eine erfindungsgemäße Indikation für das Präparat ist beispielsweise eine Behandlung eines Patienten mit einem phänotypischen Gerinnungsfaktormangel, z.B. eines vWF-defizienten Patienten. Dabei wird erfindungsgemäß ein Präparat hergestellt auf Basis des Gerinnungsfaktors, der im Patienten mangelhaft ist, mit der erfindungsgemäßen Kombination des Rezeptor-Bindungskompetitors. Aber auch der Rezeptor-Bindungskompetitor alleine entspricht der erfindungsgemäßen Indikation. Beispielsweise wird bei einem Mangel an funktionellem Faktor VIII der Rezeptor-Bindungskompetitor für Faktor VIII verabreicht, welcher eventuell das Gemisch tPA und Aprotinin ist.

Gegenstand der vorliegenden Erfindung ist auch die Verwendung einer LRP-Ligandenpräparation zur Herstellung eines Mittels zur Behandlung von phänotypischem Gerinnungsfaktormangel (z.B. vWF-, Faktor VIII- oder Faktor IX-Mangel) oder zur Verlängerung der biologischen Halbwertszeit eines Proteins.

Die Erfindung wird an Hand der nachfolgenden Beispiele und der Zeichnungen, auf die sie jedoch nicht beschränkt sein soll, näher erläutert.

Es zeigen:

Fig. 1: Faktor VIII-Bestimmung im vWF-defizienten Hund

Fig. 2: vWF und Faktor VIII-Bestimmung in vWF-defizientem Hund

B e i s p i e l e :

B e i s p i e l 1 :

Ein Kooiker Hund mit schwerem von Willebrand Faktor-Mangel vom Typ 3 (Rieger et al., Thromb. Haemost. (1998), 80: 332-337) männlichen Geschlechts, 8,5 kg Körpergewicht, 2 Jahre alt, wurde mit 10 mg/kg Körpergewicht Ketamin (Ketasol, Dr. E. Gräub AG, Bern, Schweiz) und 1 mg/kg Xylaxin (Xylasol, Dr. E. Gräub AG, Bern, Schweiz) narkotisiert. Anschließend wurde ein permanenter venöser Zugang über einen Unterarm geschaffen und über eine Kanüle Aprotinin (Pantinol 100.000 K.I.E. Ampullen, Gerot Pharmazeutika, Wien) in einer Dosis von 10 000 K.I.E. pro kg Körpergewicht als Bolus verabreicht. Anschließend wurde rekombinanter Gewebeplasminogenaktivator (Actilyse, Boehringer Mannheim) (rtPA) gemäß Herstellerangabe mit destilliertem Wasser rekonstituiert und dem von Willebrand-defizienten Hund in einer Dosis von 0,25 mg/kg Körpergewicht als Bolus verabreicht. Gleichzeitig wurde eine Blutprobe auf Zitrat entnommen. Innerhalb der ersten Stunde der Behandlung wurde eine Coinfusion von Aprotinin (3000 K.I.E. pro kg Körpergewicht) und rtPA 0,75 mg/kg Körpergewicht verabreicht und nach einer Stunde wurde auf eine Infusion gewechselt, die Aprotinin in einer Dosis von 3000 K.I.E. pro kg Körpergewicht und rtPA von 0,5 mg/kg Körpergewicht enthielt, und diese über eine Stunde verabreicht. Anschließend, d.h. nach zwei Stunden, wurde über eine weitere Stunde Aprotinin in einer Dosis von 3000 K.I.E./kg Körpergewicht infundiert. Zu den Zeitpunkten 30 min, 1, 3, 24, 48, 92 und 96 Stunden nach Versuchsbeginn wurden Blutproben auf Zitrat abgenommen und durch Zentrifugation daraus plättchenarmes Plasma hergestellt und bei -20°C tiefgefroren und bis zur weiteren Analyse gelagert.

In den gefrorenen Blutproben wurde nach Abschluß des Infusionsexperimentes Blutgerinnungsfaktor VIII mit zwei unterschiedlichen Bestimmungsmethoden quantitativ bestimmt. Erstens wurde die Zweistufengerinnungsmethode gemäß der Methode von Austen, D.E.G. und Rhymes, I.L., A Laboratory Manual of Blood Coagulation, Oxford, UK, Blackwell Scientific, (1975), unter Verwendung der Reagentien des Zweistufen-Faktor VIII - Test-Kits der IMMUNO AG, Wien, verwendet. Zweitens wurde Faktor VIII mit dem chromogenen

Faktor VIII-Test-Kit, Immunochrom FVIII:C der Immuno AG, Wien, bestimmt (Lang H., Oberreiter M., Moritz, B. Thromb. Haemost. 65:943, (1991)). Faktor VIII wurde gegen einen humanen Plasma Faktor VIII-Standard, der gegen den dritten internationalen Standard 91/666 kalibriert worden war, gemessen. Die Faktor VIII-Aktivität wird in humanen Faktor VIII-Einheiten pro ml ausgedrückt. Das Resultat dieser Bestimmung ist in der Fig. 1 zusammengefaßt. In den beiden unabhängig voneinander durchgeführten Faktor VIII-Bestimmungsmethoden zeigte sich ein Anstieg der Faktor VIII-Plasmaaktivität, der über einen Zeitraum von 24 bis 48 Stunden anhielt und anschließend auf einem Niveau von ca. 150 % des Ausgangswertes, unabhängig von der verwendeten Testmethode, über einen Zeitraum bis zu 96 Stunden nach Versuchsbeginn konstant blieb. Da bekannt ist, daß von Willebrand-Faktor gegen den proteolytischen Abbau durch Plasmin empfindlich ist, mußte angenommen werden, daß die Gabe von rtPA eine vermehrte von Willebrand-Faktor-Inaktivierung, verbunden mit einer sekundären Reduktion des Plasma-Faktor VIII-Spiegels durch Fehlen des Stabilisierungsproteins für Faktor VIII zu finden sein wird. Die gleichzeitige Gabe von Aprotinin als Inhibitor der Fibrinolyse und des Plasmins ließ lediglich eine Inhibition des rtPA-Effektes erwarten. Der scheinbar paradoxe Anstieg des Plasma-Faktor VIII-Levels kann daher durch die Interferenz von Aprotinin und/oder rtPA mit den Metabolisierungsmechanismen für Faktor VIII erklärt werden.

B e i s p i e l 2 :

Ein von von Willebrand-Faktor-defizienter Hund wie in Beispiel 1 wurde narkotisiert und anschließend mit Aprotinin (Pantinol) mit einer Dosis von 100.000 K.I.E. als intravenöser Bolus vorbehandelt. Anschließend wurde dem Tier ein rekombinantes von Willebrand-Faktor-Präparat in einer Dosis von 70 RcoF E/kg gegeben. Die Herstellung und Charakterisierung des rekombinanten von Willebrand-Faktor-Präparates ist in Fischer et al., FEBS Lett. 375:259 (1995), beschrieben. Unmittelbar nach der Gabe des rekombinanten von Willebrand-Faktor-Präparates wurde über drei Stunden eine Infusion von 100.000 K.I.E. Aprotinin intravenös verabreicht. Blutproben auf Zitrat wurden vor Beginn des Versuches (= Zeitpunkt 0) 15 min, 30 min, 1 h, 3 h, 24 h und 48 h

nach der Behandlung mit rekombinantem von Willebrand-Faktor entnommen und daraus Plasma, wie in Beispiel 1 beschrieben, hergestellt und bis zur weiteren Analyse bei -20°C tiefgefroren. Von diesen Blutproben wurde anschließend von Willebrand-Faktor-Antigen unter Verwendung eines Kaninchen Anti-Human-von Willebrand-Faktor-Antikörpers mit dem Test Asserachrom vWF, Boehringer Mannheim, gemessen. Außerdem wurde die Ristocetin-Cofaktor-Aktivität, gemessen durch Ristocetin induzierte Aggregation Formaldehyd-fixierter humaner Blutplättchen und nach der Methode von Evans und Austen, Brit. J. Hetamol. 37:289 (1977) bestimmt. Als von Willebrand-Faktor-Standard diente ein humaner Plasma-Standard. Außerdem wurde Faktor VIII, wie in Beispiel 1 beschrieben, getestet. Die Resultate der Bestimmung der Blutproben an von Willebrand-Faktor und Faktor VIII-Aktivität sind der Fig. 2 zu entnehmen. In der Grafik ist ein Mittelwert der Faktor VIII-Aktivität, gemessen nach den beiden Faktor VIII-Bestimmungsmethoden, als Graph eingezeichnet. Von Willebrand-Faktor wurde, wie aus der Literatur bekannt (Turecek et al., Blood 90: 3555 (1997)), mit einer Halbwertszeits von 10 bis 20 h eliminiert, so daß nach 48 h kaum mehr von Willebrand-Faktor in der Zirkulation nachweisbar war. Gleichzeitig mit Gabe des rekombinanten von Willebrand-Faktors und des Aprotinins stieg der Plasma-Faktor VIII-Spiegel innerhalb von 3 h auf ca. 150 % des Ausgangswertes an und blieb dann auf diesem Niveau stabil oder zeigte sogar einen leicht steigenden Trend bis zu 48 h nach der Verabreichung. Auch dieses Ergebnis kann wie Beispiel 1 interpretiert werden, daß durch die Gabe des Aprotinins trotz fehlender Stabilisierung des Faktor VIII durch den rekombinanten von Willebrand-Faktor, durch eine Interferenz mit dem Faktor VIII-Metabolismus der Faktor VIII-Plasma-Spiegel anhaltend erhöht bleibt.

B e i s p i e l 3 :

Wirkung von RAP auf Faktor VIII-Gewinnung in Knock-out-Mäusen mit schwerem Faktor VIII-Mangel

Auf gentechnischem Wege wurde ein Mäusestamm mit schwerem Faktor VIII (FVIII)-Mangel durch gezielte Disruption des Mäuse-Faktor VIII-Gens gemäß Bi et al. Nature Genetics 10:119-121 (1995) geschaffen. Faktor VIII-Knock-out-Mäuse wurden durch eine Inser-

tion eines neo-Gens in das 3'-Ende von Exon 17 des Mäuse-Faktor VIII-Gens geschaffen. Die betroffenen männlichen Tiere (X'Y) hatten nicht nachweisbare Faktor VIII-Levels von <0,02 ± 0,01 E/ml, wenn Messungen entweder mit einem chromogenen Faktor VIII-Test, Hyland Immuno, Wien, Österreich, wie kürzlich beschrieben (Turecek et al., Thromb. Haemostas. Suppl. 769 (1997)) oder mittels Antigen-ELISA, wie nachstehend beschrieben, vorgenommen wurden.

Zwei betroffene, hemizygote männliche Mäuse (X'Y) wurden intravenös mit einer Dosis von 200 E/kg Körpergewicht einer rekombinanten Human-Faktor VIII (rhFVIII)-Präparation behandelt, die von Chinese Hamster-Ovarien-Zellen stammte, die wie beschrieben hergestellt worden waren (PCT WO/85/01961), und ohne stabilisierendes Protein pharmazeutisch formuliert worden war.

Unter Narkose wurden eine Stunde nach der Behandlung die Schwanzspitzen der Mäuse mit der Klinge eines Skalpells, wie von Novak et al., Brit. J. Haematol. 69:371-378 (1998) beschrieben, eingeschnitten. Ein Volumen von 50 µl Blut wurde mit Kapillarröhrchen (Ringcaps, Hirschmann, Deutschland) aus den Schwanzwunden gesammelt, wobei die Kapillarröhrchen mit Lithium-Heparin als Antikoagulans beschichtet waren. Die Kapillarröhrchen wurden geschlossen und zentrifugiert, um die Blutzellen vom Plasma zu trennen. Danach wurden die Kapillarröhrchen geöffnet, und die Zell- und die Plasma-Fraktion wurden durch weiteres Zentrifugieren gesammelt. Schließlich wurden die Plasmaproben der Faktor VIII-Bestimmung mittels Faktor VIII-Antigen ELISA, Test-Set IMMUNOZYM FVIII Ag, Hyland Immuno, Wien, Österreich, unter Verwendung von monoklonalen Antifaktor VIII-Antikörpern sowohl für das Abfangen wie für den Nachweis, wie beschrieben (Stel et al., Nature 303:530-532 (1983); Lenting et al., J. Biol. Chem. 269: 7150-7155 (1994); Leyte et al., Biochem. J. 263:187-194 (1989)) unterzogen. Die resultierenden Faktor VIII-Werte wurden in internationalen Einheiten von humanem Faktor VIII ausgedrückt. Die Ergebnisse der Faktor VIII-Plasma-Level sind in der Tabelle angegeben.

Zwei andere betroffene hemizygote männliche Mäuse (X'Y) wurden

mit rekombinantem Rezeptor-assoziiertem Protein (GST-RAP) 10 min vor der Behandlung mit dem rekombinanten humanen Faktor VIII mit einer Dosis von 40 mg/kg Körpergewicht vorbehandelt. Das bei dieser Untersuchung verwendete Rezeptor-verwandte Protein (RAP), welches mit dem Low-Density-Lipoprotein-Rezeptor in Wechselwirkung tritt, wurde durch bakterielle Fermentation, wie von Hertz et al., J. Biol. Chem. 266:21232-21238 (1991) beschrieben, erhalten. Ein Fusionsprotein des RAP mit Glutathion-S-Transferase wurde in E. coli exprimiert und mittels Affinitätschromatographie auf Glutathion-Agarose gereinigt. Das resultierende Protein bestand hauptsächlich aus dem Fusionsprotein und Spaltprodukten von RAP und Glutathion-S-Transferase. Das Fusionsprotein wurde in einem injizierbaren Puffer bereit für eine Verabreichung an die Faktor VIII-Knock-out-Mäuse formuliert. Wie bei der Kontrollgruppe (Behandlung nur mit Faktor VIII) wurden Blutproben eine Stunde nach der Verabreichung von rekombinantem Faktor VIII genommen und unter Verwendung von Faktor VIII-Antigen-ELISA auf Faktor VIII-Aktivität gemessen. Die Ergebnisse sind in der Tabelle angeführt.

	Behandlung	Dosis	Gewinnung 1 h nach Behandlung
Maus Nr.	GST-RAP	rhFVIII	FVIII:Ag (E/ml Plasma)
1	40 mg/kg	200 E/kg	1,92
2	40 mg/kg	200 E/kg	1,88
3	-	200 E/kg	0,73
4	-	200 E/kg	0,83

Bei mit GST-RAP vorbehandelten Mäusen betrug die Faktor VIII-Gewinnung mehr als 200 % der Plasma-Level nach der Behandlung mit rekombinantem Faktor VIII allein.

P a t e n t a n s p r ü c h e :

1. Pharmazeutische Präparation zur Behandlung von Blutgerinnungsstörungen, enthaltend mindestens ein Protein, ausgesucht aus der Gruppe von einem pro-Protein der Blutgerinnung und weiters einen gerinnungsphysiologisch inerten Rezeptor-Bindungskompetitor.

2. Präparation nach Anspruch 1, dadurch gekennzeichnet, daß das pro-Protein der Blutgerinnung ausgesucht ist aus der Gruppe von Faktor II, V, VII, VIII, IX, X, XI, XII, vWF und Protein C.

3. Präparation nach Anspruch 1, dadurch gekennzeichnet, daß das Protein vWF ist.

4. Präparation nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß das Protein aus einem biologischen Material ausgesucht aus der Gruppe von Humanplasma, einer Plasmafraktion und einem Zellkulturüberstand gewonnen ist.

5. Präparation nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß der Rezeptor-Bindungskompetitor ein Ligand des Lipoprotein-Rezeptor-Related-Proteins (LRP) ist.

6. Präparation nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Rezeptor-Bindungskompetitor RAP ist.

7. Präparation nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß der Rezeptor-Bindungskompetitor ein Gemisch aus einem gerinnungsphysiologisch aktivem Protein und dessen Inhibitor ist, insbesondere tPA und Aprotinin.

8. Präparation nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, daß das Gemisch tPA und Aprotinin enthält.

9. Präparation nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß es als Set zur Verfügung gestellt wird, enthaltend

a) das Protein und

b) den Rezeptor-Bindungskompetitor.

10. Präparation nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß das Protein Faktor VIII ist und der Rezeptor-Bindungskompetitor Aprotinin, gegebenenfalls gemeinsam mit tPA.

11. Präparation nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß das Protein vWF ist und der Rezeptor-Bindungskompetitor Aprotinin ist.

12. Präparation nach einem der Ansprüche 1 bis 11, dadurch gekennzeichnet, daß der Rezeptor-Bindungskompetitor ein humanphysiologisches extrazelluläres Protein ist, oder dessen Analoges.

13. Präparation nach einem der Ansprüche 1 bis 12, dadurch gekennzeichnet, daß der Rezeptor-Bindungskompetitor ausgewählt aus der Gruppe der Enzyme, insbesondere Tissue-type Plasminogenaktivator (tPA), Urokinase (u-PA), pro-Urokinase (pro-u-PA), Lipoproteinlipase (LPL) und Kallikrein; Inhibitoren, insbesondere Plasminogen-Aktivator-Inhibitor-1 (PAI-1), Tissue factor pathway Inhibitor (TFPI) und Aprotinin; Enzym-Inhibitor-Komplexe, insbesondere t-PA-PAI-1, u-PA-PAI-1 und Thrombin-PAI-1, α_2-Macroglobulin-Proteinase (fast form) α_2M, α_2M^*, Pregnancy zone protein-Proteinase, Elastase-α^1-Antitrypsin, Thrombin-Antithrombin III, Thrombin-Heparin-Cofactor II und u-PA-Protease-Nexin I; Lipoproteine, insbesondere low density-Lipoprotein (LDL), Apolipoprotein E angereichertes β-very low density-Lipoprotein, (apo E-β-VLDL), LPL angereichertes VLDL (LPL-VLDL) und LPL angereichertes β-VLDL (LPL-β-VLDL); Matrix-Proteine, insbesondere Thrombospondin 1 und 2; Toxine und Viren, insbesondere Pseudomonas Exotoxin A, und Minor-group human Rhinovirus; oder andere Liganden, insbesondere Apolipoprotein E (apo E), Lactoferrin, Rezeptor assoziiertes Protein (RAP), FVIII und vWF, ist.

14. Kombinationspräparat enthaltend Aprotinin und tPA zur medizinischen Verwendung.

15. Verwendung eines Präparates nach einem der Ansprüche 1 bis 13 zur Herstellung eines Mittels zur Behandlung eines Patienten

mit einem phänotypischen Gerinnungsfaktor-Mangel.

16. Verwendung nach Anspruch 15, dadurch gekennzeichnet, daß der Patient vWF-defizient ist.

17. Verwendung eines Präparates nach einem der Ansprüche 1 bis 13 zur Herstellung eines Mittels zur Verlängerung der biologischen Halbwertszeit des Proteins in vivo.

18. Verwendung eines Präparates nach Anspruch 17 enthaltend Aprotinin und tPA.

19. Verwendung nach Anspruch 17, dadurch gekennzeichnet, daß das Protein Blutgerinnungsfaktor VIII ist.

20. Verwendung einer pharmazeutischen Präparation eines LRP-Liganden zur Herstellung eines Mittels zur Behandlung eines Patienten mit phänotypischem Gerinnungsfaktor-Mangel.

21. Verwendung eines Präparates nach Anspruch 20 enthaltend Aprotinin und tPA.

22. Verwendung nach Anspruch 20, dadurch gekennzeichnet, daß der Patient vWF-defizient ist.

23. Verwendung einer pharmazeutischen Präparation eines LRP-Liganden zur Herstellung eines Mittels zur Verlängerung der biologischen Halbwertszeit eines Proteins.

24. Verwendung einer pharmazeutischen Präparation nach Anspruch 23 enthaltend Aprotinin und tPA.

25. Verwendung nach Anspruch 23, dadurch gekennzeichnet, daß das Protein Blutgerinnungsfaktor VIII ist.

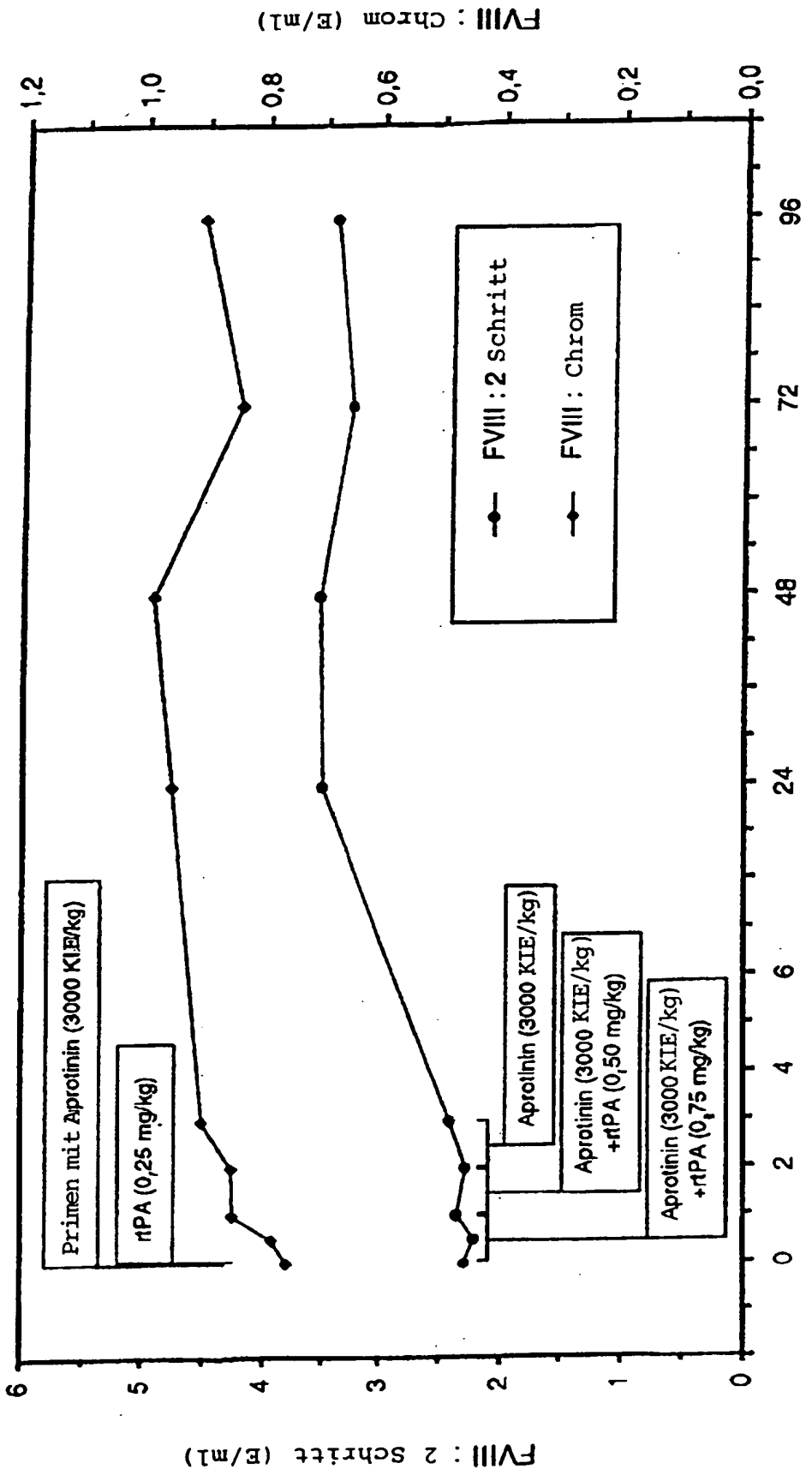

Wirkung von Aprotinin und rtPA bei einem vWF-defizienten Hund
FVIII : 2 Schritte (E/ml)
FVIII : Chrom (E/ml)
Zeit (h)
FVIII : 2 Schritt
FVIII : Chrom
Primen mit Aprotinin (3000 KIE/kg)
rtPA (0,25 mg/kg)
Aprotinin (3000 KIE/kg)
Aprotinin (3000 KIE/kg) +rtPA (0,50 mg/kg)
Aprotinin (3000 KIE/kg) +rtPA (0,75 mg/kg)

FIG.1

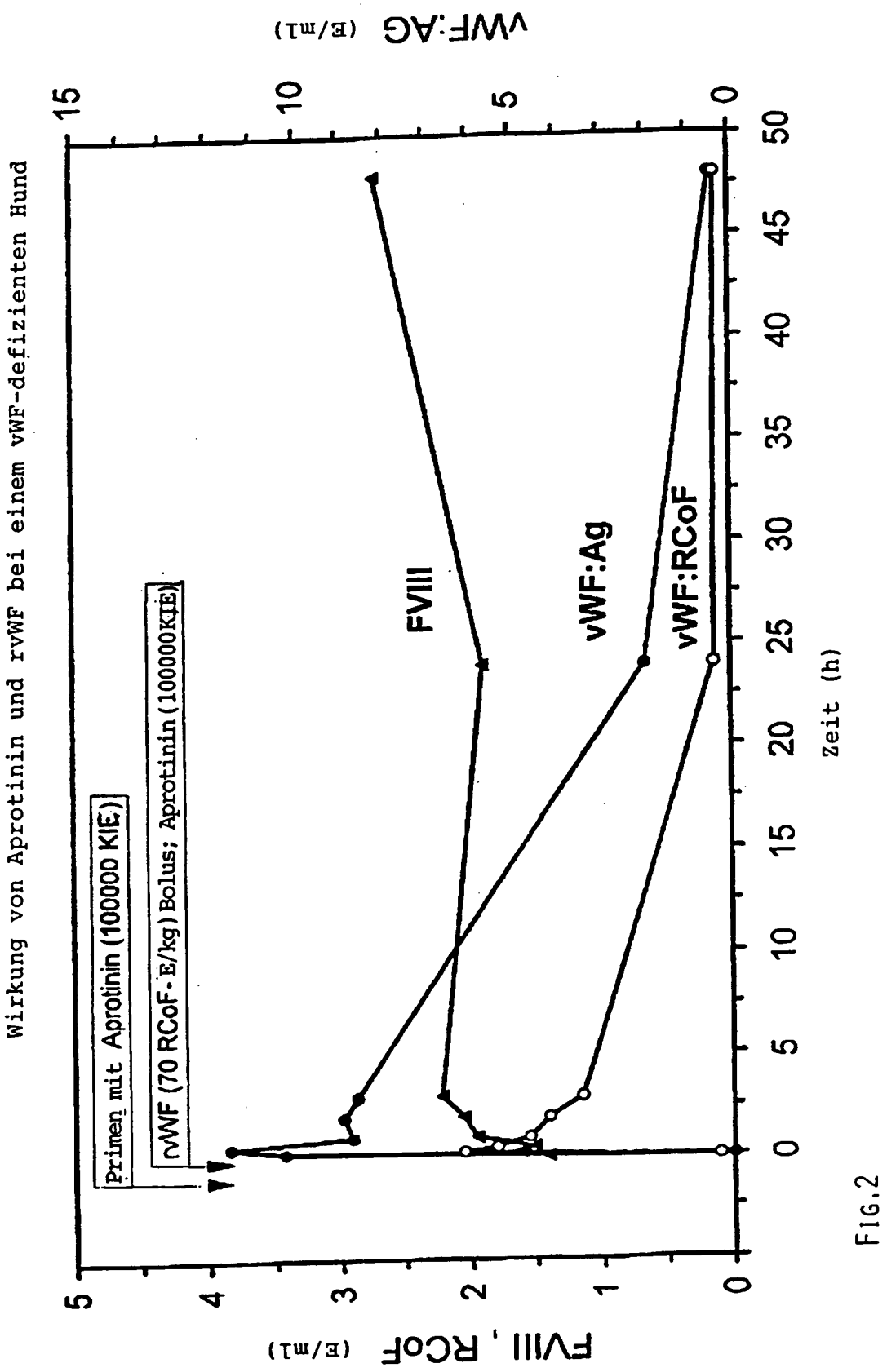

Wirkung von Aprotinin und rvWF bei einem vWF-defizienten Hund
Primen mit Aprotinin (100000 KIE)
rvWF (70 RCoF-E/kg) Bolus; Aprotinin (100000KIE)
FVIII
vWF:Ag
vWF:RCoF
FVIII , RCoF (E/ml)
vWF:AG (E/ml)
Zeit (h)
0
1
2
3
4
5
0
5
10
15
0
5
10
15
20
25
30
35
40
45
50

FIG.2

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/47, 37/43, C07H 21/00

A1

(11) International Publication Number: WO 94/14471

(43) International Publication Date: 7 July 1994 (07.07.94)

(21) International Application Number: PCT/US93/12380

(22) International Filing Date: 17 December 1993 (17.12.93)

(30) Priority Data:
07/992,827 18 December 1992 (18.12.92) US

(71) Applicant: WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US).

(72) Inventors: SCHWARTZ, Alan, L.; 11 Southmoor Drive, Clayton, MO 63105 (US). BU, Guojun; 1821 Canyon View Court, Chesterfield, MO 63017 (US).

(74) Agent: BARNHARD, Elizabeth, M.; Bryan Cave, 245 Park Avenue, New York, NY 10167-0034 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

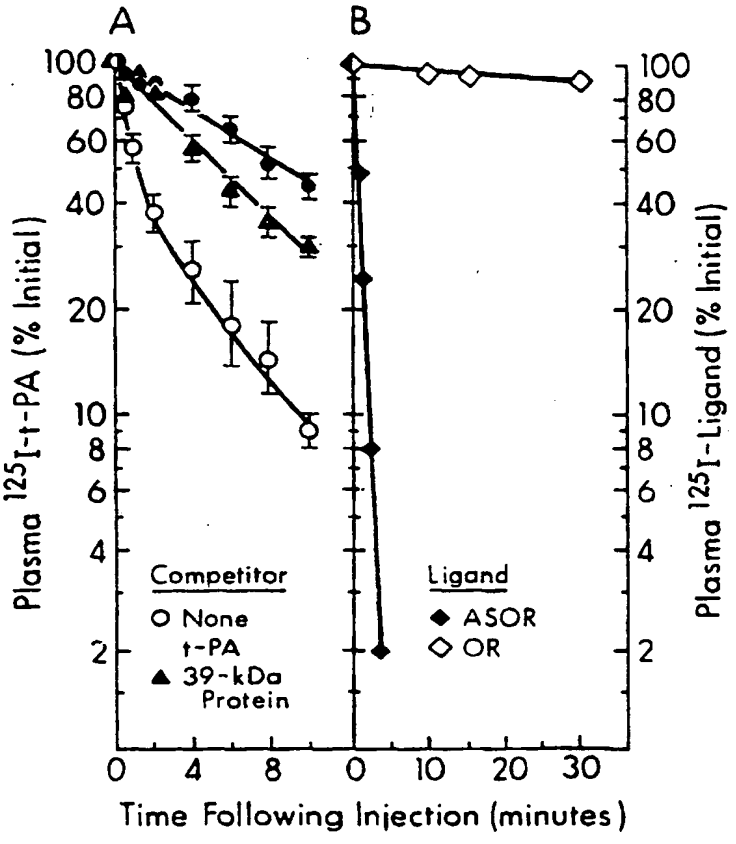
Published
With international search report.
With amended claims.

Date of publication of the amended claims:
4 August 1994 (04.08.94)

(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

(57) Abstract

Methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* by administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof are described. Compositions for treatment of thrombolytic diseases comprised of t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein, a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments are described.



* (Referred to in PCT Gazette No. 21/1994, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

TITLE

METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

TECHNICAL BACKGROUND

This invention was made with U.S. Government support under HL 17646 awarded by the National Institutes of Health. The U.S. Government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* comprising administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof; and to methods and compositions for treatment of thrombolytic diseases comprising administering t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein, a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments.

BACKGROUND OF THE INVENTION

Tissue plasminogen activator is an enzyme widely used as a thrombolytic agent in the treatment of acute myocardial infarction. t-PA is secreted from endothelial cells as a single polypeptide chain that is subsequently cleaved (between Arg_{275} and Ile_{276}) into two chains held together by a single disulfide bond (Rijken, D.C. et al., (1981) J. Biol. Chem. 256, 7035-7041. Both the single- and two-chain forms of the enzyme can bind to fibrin (Rijken, D.C. et al., (1982) J. Biol. Chem. 257, 2920-

2925; Higgins, D.L. et al., (1987) Biochem. 26, 7786-7791), although the two-chain form is catalytically more active (Wallen, P. et al., (1982) Biochim. Biophys. Acta 719, 318-328; Ranby, M., (1982) Biochim Biophys. Acta 704, 461-469; Tate, K.M. et al., (1987) Biochem. 26, 338-343; and Petersen, L.C. et al., (1988) Biochim. Biophys. Acta 952, 245-254). In addition, both forms of the enzyme (Jorgensen, M. et al., (1987) Thromb. Haemostasis 58, 872-878) are inactivated by a fast-acting plasminogen activator inhibitor (type 1)(PAI-1), a member of the serpin family that is secreted from endothelial cells and forms a covalent bond with Ser_{478} of t-PA (Levin, E.G., (1983) Proc. Natl. Acad. Sci. USA 80, 6804-6808; and Sprengers, E.D. et al., (1987) Blood 69, 381-387).

Exogenously administered t-PA is capable of eliciting prompt thrombolysis in therapeutic doses that do not produce marked fibrinolysis in experimental animals with induced coronary artery thrombosis (Bergmann, S.R. et al., (1983) Science, 220, 1181-1183) and in patients with evolving myocardial infarction (Van de Werf, F. et al., (1984) New Eng. J. Med. 310, 609-613; Collen, D. et al., (1984) Circ. 70, 1012-1017; Van de Werf, F. et al., (1984) Circ. 69, 605-610; Thrombolysis in Myocardial Infarction Study Group, (1985) New Eng. J. Med. 312, 932-936; and European Cooperative Study Group, (1985) Lancet 1, 842-847). However, because the clearance of t-PA from the circulation is so rapid, continuous infusions have been required.

In vivo studies of t-PA clearance have been performed in a variety of species including mice (Fuchs, H.E. et al.,

(1985) Blood 65, 539-544); rats (Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986) Biochem. J. 238, 643-646; Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224; Bakhit, C. et al., (1988) Fibrin. 2, 31-36; Krause, J. et al., (1990) Biochem. J. 267, 647-652); rabbits (Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-661; Bounameaux, H. et al., (1986) Blood 67, 1493-1497); dogs (Devries, S.R. et al., (1987) Fibrin. 1, 17-21; Yasuda, T. et al., (1988) J. Clin. Invest. 81, 1284-1291); and monkeys (Flameng, W. et al., (1985) J. Clin. Invest. 75, 84-90). These studies together with experiments in man (Garabedian, H.D. et al., (1986) Am. J. Cardiol. 58, 673-679; Verstraete, M. et al., (1986) Thromb. Haemostas. 56, 1-15) demonstrate the rapid removal of t-PA from the circulation, which varies from about $t_{1/2}$ = 1 min in rats to $t_{1/2}$ = 5 min in man.

The liver appears to be the major site of removal and catabolism of t-PA (Nilsson, T. et al., (1984) Scand. J. Haematol. 33, 49-53; Devries, S.R. et al., (1987) Fibrin. 1, 17-21; Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-661; Bounameaux, H. et al., (1986) Blood 67, 1493-1497; Beebe, D.P. et al., (1986) Thromb. Res. 43, 663-674; Nilsson, S. et al., (1985) Thromb. Res. 39, 511-521; Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986) Biochem. J. 238, 643-646; Fuchs, H.E. et al., (1985) Blood 65, 539-544; and Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224). About 80% of exogenous t-PA delivered intravascularly rapidly accumulates in the liver and is subsequently degraded, with subsequent appearance of degradation

products in plasma. These studies support a general clearance mechanism for t-PA in which uptake and degradation within the liver is followed by the release of the degradation products initially into the blood and subsequently into the urine.

Further, the half-life of circulating t-PA is markedly prolonged in animals subjected to hepatectomy (Bounameaux, H. et al., (1986) Blood 67, 1493-1497; and Nilsson, T. et al., (1984) Scand. J. Haematol. 33, 49-53). Neither the protease active site nor a specific glycosylation pattern appears to be a major determinant of hepatic recognition and degradation of t-PA *in vivo* (Fuchs, H.E. et al., (1985) Blood 65, 539-544), in perfused liver systems (Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664, or in isolated hepatocytes (Bakhit, C. et al., (1987) J. Biol. Chem. 262, 8716-8720. The clearance and catabolism of t-PA has been reviewed in detail (Krause, J. (1988) Fibrin. 2, 133-142). However, information is limited regarding the particular cell type responsible for clearance of t-PA.

After administration of fluorescent or radiolabelled t-PA to rats and subfractionation of the livers into parenchymal, endothelial, and Kupffer cells, it was found that parenchymal and endothelial cells constitute the major sites for hepatic uptake (Fuchs, H.E. et al., (1985) Blood 65, 539-544; Sprengers, E.D. et al., (1987) Blood 69, 381-387; Kuiper, J. et al., Fibrin, 2:28 (1988) and Bugelski, P.J. et al., (1989) Thromb. Res. 53, 287-303).

The uptake of t-PA into all liver cell types is inhibited by *in vivo* competition with unlabelled t-PA whereas glycoproteins such as mannan and ovalbumin inhibit the specific

uptake of labelled t-PA in isolated liver endothelial cells. The endocytosis of t-PA is mediated, at least in part, by mannose receptors on endothelial cells (Einarsson, M. et al., (1985) Thromb. Haemost. 54, 270; and Kuiper, J. et al., (1988) Fibrin. 2, 28). Monensin, NH_4Cl, and cytochalasin B block the uptake and degradation of t-PA, indicating that the uptake is endocytotic and that the degradation is lysosomal. In hepatoma cell lines, representing parenchymal cells, t-PA clearance involves ligand binding, uptake, and degradation mediated by a high capacity, high-affinity specific receptor system (Owensby, D.A. et al., (1988) J. Biol. Chem. 263, 10587-10594). Subfractionation of rat liver parenchymal, endothelial, and Kupffer cells 5 minutes after ^{125}I-t-PA injection revealed that liver parenchymal cells are responsible for about 55% of the cleared ^{125}I-t-PA, endothelial cells for about 40%, and Kupffer cells for about 5% (Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224; Rijken, D.C. et al., (1990) Thromb. Res. Suppl. X, 63-71).

Two distinct mechanisms for t-PA catabolism by hepatoma cells have been shown. t-PA complexed to PAI-1 is recognized by a PAI-1 dependent receptor on the cell surface of human hepatoma HepG2 cells (Schwartz, A.L. et al., (1981) J. Biol. Chem. 256, 8878-8881; Owensby, D.A. et al., (1988) J. Biol. Chem. 263, 10587-10594; Morton, P.A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). t-PA in the absence of bioactive PAI-1 has been found to bind to PAI-1 independent receptors which mediate binding and endocytosis of t-PA on rat hepatoma MH_1C_1 cells (Bu,

G. et al., (1992) J. Biol. Chem. 267, 15595-15602) and on rat Novikoff hepatoma cells (Nguyen, G. et al., (1992) J. Biol. Chem. 267, 6249-6256). Although this PAI-1 independent t-PA clearance system has not been reported on human hepatocytes, the rapid clearance of intravenously injected t-PA, normally at a level far exceeding the available PAI-1, suggests the existence of a PAI-1 independent t-PA clearance system.

At present, t-PA is administered clinically in the form of an initial bolus that is followed by sustained infusion. The total amount of enzyme administered during a standard 3 hour treatment is 50-100 mg. Such large amounts are required for two reasons: first, to counterbalance the effects of the rapid clearance of t-PA from the circulation, and second, to overcome the effects of high concentrations of fast-acting inhibitors of the enzyme that are present in plasma and platelets. When high doses are used in an effort to increase the rate of clot lysis or to lyse refractory clots, there is a risk of systemic fibrinolysis which affects the body's capacity to stop bleeding and hemorrhage.

Reducing the rate of t-PA clearance from the circulation following administration would provide a significant advantage in clinical use. This would allow t-PA to be administered in much smaller doses than are currently required, thereby reducing, e.g., the risk of systemic fibrinolysis and hemorrhage. Accordingly, an objective of the invention is to provide methods and compositions for the inhibition of hepatic clearance of t-PA. Another objective of the invention is to provide a method and composition for the treatment of

thrombolytic diseases which allows t-PA to be administered in much smaller doses than currently required. These and other objectives and f atures of the invention will be apparent from the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph of the saturation binding of ^{125}I-39kDa protein to MH_1C_1 cells in the presence or absence of 0.5μM unlabeLled 39kDa protein. (*circles*) = total ligand binding; (*triangles*) = non-specific ligand binding. Specific binding (*squares*) was calculated as the difference between total and non-specific binding. Each symbol represents the mean of triplicate determinations. The *inset* is a Scatchard plot of specific binding where B = bound ^{125}I-39kDa protein and B/F = bound/free ^{125}I-39kDa protein. FIG. 1B is a SDS-PAGE gel (10% acrylamide, non-reduced) of equivalent volumes of post-binding cell lysates, each from about 60,000 cells. The position of ^{125}I-39kDa protein is indicated by a closed arrow.

FIGS. 2A-B are graphs of the saturation binding of ^{125}I-t-PA and ^{125}I-39kDa protein to HepG2 cells. FIG. 2A is a graph of the saturation binding of ^{125}I-t-PA in the absence or presence of 1 μM unlabelled t-PA. (*squares*) = total ligand binding; (*triangles*) = non-specific ligand binding; (*circles*) = specific binding calculated as the difference between total and non-specific binding. *Symbols* represent the means of triplicate determinations. The *inset* is a Scatchard plot of specific binding, where B = bound ^{125}I-t-PA, B/F = bound/free ^{125}I-t-PA. FIG. 2B is a graph of the saturation binding of the ^{125}I-39kDa

protein. The same symbols are used as in FIG. 2A. The *inset*, like FIG. 2A, is a Scatchard plot of specific binding.

FIGS. 3A-D are SDS-PAGE gels (8.5% acrylamide) of specific cross-linking of ^{125}I-39kDa protein to low density lipoprotein receptor-related protein (LRP) in the presence and absence of 0.5μM unlabelled 39kDa protein. In FIGS. 3A and 3B, MH_1C_1 cells were incubated in the presence of 0.5mM DTSSP for 1 hour. In FIGS. 3C and 3D, MH_1C_1 cells were incubated in the absence of 0.5mM DTSSP for 1 hour. Cells were then lysed and immunoprecipitated with normal rabbit serum (*lanes 1 and 4*), anti-LRP serum (*lanes 2 and 5*), or anti-39kDa protein serum (*lanes 3 and 6*). In FIGS. 3A and 3C, the SDS-PAGE gels were analyzed under nonreducing conditions. In FIGS. 3B and 3D, the SDS-PAGE gels were analyzed under reducing conditions. Gels from samples incubated in the presence of cross-linker were autoradiographed for 3 days at -70°C; gels incubated in the absence of cross-linker were autoradiographed for only 2 days. The position of ^{125}I-39kDa protein (*closed arrowhead*) is indicated.

FIGS. 4A-B are SDS-PAGE gels of chemical cross-linking of ^{125}I-t-PA:PAI-1 to LRP on HepG2 cells. (N.R.) = normal rabbit serum; (α-t-PA) = anti-t-PA antibody; (α-PAI-1) = anti-PAI-1 antibody; (α-LRP) = anti-LRP antibody. FIG. 4A is a SDS-PAGE gel (7.5% acrylamide) analyzed under nonreducing conditions. FIG. 4B is a SDS-PAGE gel (7.5% acrylamide) analyzed under reducing conditions. The region of cross-linked material is marked with a bracket. The positions of ^{125}I-t-PA (*closed arrow*) and ^{125}I-t-PA:PAI-1 (*open arrow*) are indicated.

FIG. 5A is a graph of the inhibition of 3 nM ^{125}I-t-PA binding to H pG2 cells by the 39kDa protein in the absence or presence of increasing concentrations of competitor proteins. Each symbol represents the average of duplicate determinations and the standard deviations are less than 5%. FIG. 5B is a SDS polyacrylamide gel (7.5%) of post-binding buffers overlying cell monolayers and corresponding cell lysates where the gel was analyzed under nonreducing conditions using a sample buffer containing low concentration of SDS (0.2%). Samples were selected from either no competitor protein (lanes 1 and 5), or with 500 nM t-PA (lanes 2 and 6), 500 nM 39kDa protein (lanes 3 and 7), or 500 nM BSA (lanes 4 and 8).

FIG. 6 are SDS-PAGE gels of chemical cross-linking of t-PA:PAI-1 to LRP on ^{35}S-methionine-labelled HepG2 cells which were analyzed under nonreducing or reducing conditions. (N.R.) = normal rabbit serum (*lanes 1, 6, 11*); (α-t-PA) = anti-t-PA antibody (*lanes 2, 7, 12*); (α-PAI-1) = anti-PAI-1 antibody (*lanes 3, 8, 13*); (α-39kDa) = anti-39kDa protein antibody (*lanes 4, 9, 14*); (α-LRP) = anti-LRP antibody (*lanes 5, 10, 15*). The regions of cross-linked material in the non-reducing gel are marked with brackets. The following positions are indicated: LRP large subunit (515 kDa) (*2 closed arrowheads*); LRP small subunit (85 kDa) (*1 closed arrowhead*); PAI-1 (*1 closed arrow*); and t-PA:PAI-1 complex (*1 open arrow*).

FIGS. 7A-B are SDS-PAGE gels (8.5% acrylamide) of cross-linking of unlabelled 39kDa protein to the cell surface of metabolically labelled rat MH_1C_1 cells. Cell monolayers in lanes 4-6 w re incubated for 1 hour with 0.5 mM DTSSP, whereas cell

monolayers in lanes 1-3 were incubated for 1 hour in PBSc alone. Cells were then lysed and immunoprecipitated with either normal rabbit serum (*lanes 1 and 4*), affinity purified α-rat 39kDa protein (*lanes 2 and 5*), or affinity purified α-human LRP (*lanes 3 and 6*). In FIG. 7A, the SDS-PAGE gel was analyzed under nonreducing conditions. In FIG. 7B, the SDS-PAGE gel was analyzed under reducing conditions. The *closed arrowhead* indicates the position of the 39kDa protein. The *open* and *closed arrows* indicate, respectively, the positions of the 85kDa and 520kDa subunits of LRP.

FIGS. 8A-F are SDS-PAGE gels (8.5% acrylamide) of co-binding and cross-linking of ^{125}I-39kDa protein and unlabelled t-PA to rat MH_1C_1 cells in the presence or absence of 0.5 μM unlabelled 39kDa protein. In FIGS. 8A, 8C, and 8E, the SDS-PAGE gels were analyzed under nonreducing conditions. In FIGS. 8B, 8D, and 8F, the SDS-PAGE gels were analyzed under reducing conditions.

FIG. 9 is a graph of rapid endocytosis and degradation of surface bound ^{125}I-39kDa protein by MH_1C_1 cells in the presence and absence of 0.5 μM unlabelled 39kDa protein. (*circles*, degraded ligand) = TCA soluble radioactivity; (*diamonds*, both dissociated and degraded ligand) = total radioactivity; (*squares*, plasma membrane associated) = Pronase sensitive ligand; (*triangles*, cell associated) = Pronase resistant ligand. Each data point represents specific radioactivity (i.e., the difference of total and non-specific radioactivity) and is the mean of triplicate determinations.

FIGS. 10A-B are graphs of the inhibition of ^{125}I-t-PA uptake and degradation by the 39kDa protein on HepG2 cells. (*open squares*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM); (*open circles*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration of t-PA; (*open triangles*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration of the 39-kDa protein; (*closed circles*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration of BSA. FIG. 10A shows TCA soluble radioactivity representing the degraded ligands in fmoles equivalents of t-PA. FIG. 10B shows the radioactivity associated with each cell lysate in fmoles equivalents of t-PA. In each graph, each symbol represents the average of triplicate determinations and the standard deviations were less than 5%.

FIGS. 11A-B are graphs of the inhibition of ^{125}I-t-PA binding by rat 39kDa protein on MH_1C_1 cells in the presence of increasing concentrations of either unlabelled t-PA or 39kDa protein. Each point represents the mean of triplicate determinations. FIG. 13A shows the inhibition of ^{125}I-t-PA binding by the 39kDa protein. FIG. 13B shows the inhibition of ^{125}I-t-PA binding by unlabelled t-PA.

FIG. 12 is a graph of the rapid uptake and degradation of ^{125}I-39kDa protein by rat MH_1C_1 cells at 37°C. *Closed circles* represent TCA soluble counts (internalized and degraded ligand) in the extracellular media. *Triangles* indicate the amount of cell associated radioactivity as determined following lysis of the cell monolayers. *Open circles* represent the sum of both

cell associated and extracellular degraded ligand. Each point is the mean of triplicate determinations.

FIG. 13 is a graph of the inhibition of the ^{125}I-39kDa protein by t-PA. Each symbol represents the average of duplicate determinations and the standard deviations are less than 5%.

FIG. 14 is a graph of the inhibition of 3 nM ^{125}I-t-PA binding to HepG2 cells by anti-LRP antibody. (*open circles*) = rabbit non-immune IgG; (*closed circles*) = rabbit polyclonal anti-LRP IgG. Each symbol represents the average of duplicate determinations and the standard deviations are less than 5%.

FIG. 15A is a Coomassie-stained SDS-PAGE gel of purified 39kDa protein (10 μg). FIG. 15B is a graph of the inhibition of ^{125}I-t-PA binding to rat liver MH_1C_1 cells by 39kDa protein.

FIGS. 16A-B are graphs of the plasma clearance of ^{125}I-t-PA in rat *in vivo* in the absence or presence of unlabelled t-PA or 39kDa protein. Bars = S.E.M. FIG. 16A shows the plasma clearance of ^{125}I-t-PA alone (n=8) (*open circles*); the plasma clearance of ^{125}I-t-PA when 12 nmol unlabelled t-PA is administered prior to ^{125}I-t-PA (n=3) (*closed circles*); and the plasma clearance of ^{125}I-t-PA when 250 nmol unlabelled 39kDa protein is administered prior to ^{125}I-t-PA (n=4) (*closed triangles*). FIG. 16B shows the plasma clearance of control proteins ^{125}I-asialorosomucoid (*closed diamonds*) and ^{125}I-orosomucoid (*open diamonds*).

FIG. 17 is a graph of the plasma clearance of ^{125}I-39kDa protein in rat *in vivo* in the presence of unlabelled 39kDa

protein. (O) = administration of ^{125}I-39kDa protein alone (n=6); ( ) = administration of 12.5 nmol unlabelled 39kDa protein prior to ^{125}I-39kDa protein (n=4); (■) = administration of 50 nmol unlabelled 39kDa protein prior to ^{125}I-39kDa protein (n=4); (▲) = administration of 125 nmol unlabelled 39kDa protein prior to ^{125}I-39kDa protein (n=2).

FIG. 18 is a graph of the inhibition of ^{125}I-t-PA binding to rat liver MH_1C_1 cells by intact 39kDa protein, 20kDa N-terminal fragment and 28kDa C-terminal fragment. (●) = intact 39kDa protein; (■) = the 20kDa N-terminal fragment; (▲) = the 28kDa C-terminal fragment; and (O) = PVDF-elution buffer alone.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting the hepatic clearance of t-PA *in vivo* in humans by the administration of a t-PA-hepatic clearance-inhibiting amount of a 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof. The 39kDa protein and fragments thereof may be genetically or chemically modified.

An advantage of this method is that the plasma half-life of t-PA is significantly increased.

The present invention also provides a 28kDa protein and a chemically synthesized gene encoding this protein. It has been found that this 28kDa protein inhibits hepatic clearance of t-PA.

The present invention also provides a method of thrombolysis in a mammal by the administration of a thrombolytically effective amount of t-PA and a t-PA-hepatic

clearance-inhibiting effective amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

An advantage of this method is that the inhibition of hepatic clearance of t-PA enables smaller doses of t-PA to be used to achieve the same level of thrombolysis.

The present invention further provides a pharmaceutical composition for a mammalian patient comprising t-PA and a t-PA- hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

An advantage of this pharmaceutical composition is that the inclusion of a t-PA-hepatic clearance-inhibiting protein enables a smaller dosage amount of t-PA to be used with both physiological and cost benefits for the patient.

The present invention also provides a composition for treating thrombolytic diseases in a mammal comprising t-PA and a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

An advantage of this composition is that the amount of t-PA effective for treating thrombolytic diseases is markedly reduced in comparison to current requirements.

Upon further study of the specification and appended claims, further objectives and advantages of this invention will become apparent to those skilled in the art.

DETAILED DESCRIPTION OF THE INVENTION

It is to be understood that while the present invention is primarily contemplated for humans, it is also contemplated for use in veterinary medicine.

In accordance with the invention, a 39kDa protein which competitively binds the hepatic receptor for t-PA has been found. By binding to the hepatic receptor for t-PA, the 39kDa protein prevents t-PA from being bound to the receptor and removed from the circulating plasma by endocytosis. This increases the plasma half-life of t-PA thereby prolonging t-PA's therapeutic effectiveness. An increase in the plasma half life of t-PA means that a smaller amount of t-PA may be used, which reduces the risk of systemic fibrinolysis and hemorrhage. As t-PA is very expensive, a significant cost savings can be achieved which, in turn, increases the availability of t-PA for clinical use.

The 39kDa protein is an active, effective, competitive binding agent for the hepatic receptor for t-PA. This t-PA-hepatic clearance-inhibiting protein is characterized by binding to LRP and inhibiting the binding of t-PA to LRP up to about 80% (K_i of about 0.5 nM) (see Examples 5-8 and 10, *infra*). Fragments of this 39kDa protein, particularly a 28kDa protein fragment, also competitively bind to the t-PA hepatic receptor. When the 39kDa protein or t-PA-hepatic clearance inhibiting fragment thereof is employed in the present invention, the standard dose of t-PA is reduced up to 90%.

Hepatic clearance of t-PA is inhibited *in vivo* in humans by administering a t-PA-hepatic clearance-inhibiting amount of the 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment of the 39kDa protein. The mode of administration is preferably intravenous. The preferred amount of 39kDa protein or fragment administered to the human to

inhibit hepatic clearance is in the range of about 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the preferred amount administered to the human to inhibit hepatic clearance is in the range of about 38 to 3,800 mg/kg of body weight/dose. The 39kDa protein or its fragments may be administered to the human concurrently with t-PA, but is preferably administered up to 20 minutes prior to the administration of t-PA. Administration of the 39kDa protein or its fragments reduces hepatic clearance of t-PA between 20 and 100%. The reduction in hepatic clearance is measured by the increase in plasma half-life of t-PA.

It is to be understood that modified forms of the 39kDa protein and fragments thereof which inhibit hepatic clearance of t-PA that are made by chemically or genetically modifying the amino acid sequence of the 39kDa protein or its fragments are encompassed within the scope of the present invention. Such modified forms are characterized by their ability to bind to LRP and to reduce hepatic clearance of t-PA between 20 and 100%.

The 39kDa protein of the present invention has the following amino acid sequence set forth in the Sequence Listing as SEQ ID NO: 1:

```
1                 10                  20                  30
Y S R E K N Q P K P S P K R E S G E E F R M E K L N Q L W E K A Q

        40                  50                  60
R L H L P P V R L A E L H A D L K I Q E R D E L A W K K L K L D G

    70                  80                  90
L D E D G E K E A R L I R N L N V I L A K Y G L D G K K D A R Q V

100                 110                 120                 130
T S N S L S G T Q E D G L D D P R L E K L W H K A K T S G K F S G
```

```
                    140                 150                 160
E E L D K L W R E F L H H K E K V H E Y N V L L E T L S R T E E I

          170                 180                 190
H E N V I S P S D L S D I K G S V L H S R H T E L K E K L R S I N

  200                 210                 220                 230
Q G L D R L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q

                240                 250                 260
S A N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q K Q L

          270                 280                 290
E I A H E K L R H A E S V G D G E R V S R S R E K H A L L E G R T

    300                 310                 320
K E L G Y T V K K H L Q D L S G R I S R A R H N E L
```

The 39kDa protein was prepared as shown in Example 1.

EXAMPLE 1

Purification of the 39kDa Protein

The procedure for purification of the 39kDa protein from strains of *E.coli* carrying the over-expression plasmid *pGEX-39kDa* has been described in Herz, J. et al., (1991) J. Biol. Chem. 266, 21232-21238. A modified version of that procedure, described below, was employed.

Cultures of *E.coli* strain DH5α carrying the over-expression plasmid *pGEX-39kDa* were grown to mid-log phase in LB medium with 100 μg/ml ampicillin at 37°C. Cultures were cooled to 30°C and supplemented with 0.01% isopropylthio-β-D-galactoside to induce expression of the glutathione-S-transferase-39kDa fusion protein. Following a 4-6 hour induction at 30°C, cultures were cooled on ice and collected by centrifugation.

All of the following steps were carried out at 4°C. Cell pellets were lys d in PBSa with 1% Triton X-100, 1 μM pepstatin, 2.5 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl

fluoride (PMSF), and 1 μM ethylenediaminetetraacetate (EDTA). Sonication of this lysate was performed using a Branson Model 450 Sonifier, with the resulting membranes and other cellular debris collected by centrifugation at 15,000 *g* for 15 minutes. The supernatant from this step was incubated overnight with agarose immobilized glutathione beads (Sigma Chemical Co.) in PBSa and 0.1% sodium azide. The beads were then washed, and elution of the fusion protein was carried out by competition with 5 mM reduced glutathione (Sigma Chemical Co.). Following dialysis, the fusion protein was cleaved by an overnight incubation with 100 ng of activated human thrombin per 50 μg of fusion protein. The glutathione-S-transferase epitope was subsequently removed by further incubation with agarose immobilized glutathione beads.

* * * * *

The 28kDa protein fragment of the 39kDa protein ("28kDa protein") of the present invention has the following amino acid sequence set forth in the Sequence Listing as SEQ ID NO: 2:

```
1                 10                  20                  30
P R L E K L W H K A K T S G K F S G E E L D K L W R E F L H H K

        40                  50                  60
E K V H E Y N V L L E T L S R T E E I H E N V I S P S D L S D I K

    70                  80                  90
G S V L H S R H T E L K E K L R S I N Q G L D R L R R V S H Q G Y

  100                 110                 120                 130
S T E A E F E E P R V I D L W D L A Q S A N L T D K E L E A F R

        140                 150                 160
E E L K H F E A K I E K H N H Y Q K Q L E I A H E K L R H A E S V

            170                 180                 190
G D G E R V S R S R E K H A L L E G R T K E L G Y T V K K H L Q D
```

200 209
L S G R I S R A R H N E L

The 28kDa protein is characterized by a molecular weight of 28,000 daltons on SDS-PAGE, stability to acid hydrolysis, solubility in 1% Triton X-100, and having approximately the same inhibitory activity (K_i) on t-PA binding to the hepatic receptor as the 39kDa protein. The 28kDa protein may be cloned and purified as shown in the following example.

EXAMPLE 2

Cloning of the 28kDa Protein

The 28kDa protein is produced with a bacterial expression system. The gene encoding this protein is synthesized using polymerase chain reaction (PCR) with the following primers set forth in the Sequence Listing as SEQ ID NO: 3 and SEQ ID NO: 4, respectively:

5'CCGCGTGGATCCCCCAGGCTGGAAAAGCTGTGG3',

5'TCAATGAATTCTCAGAGTCGCTCGCCGTCGCCCAC3'.

These PCR primers contain built-in restriction sites (BamH1 and EcoR1, respectively). The PCR product after restriction enzyme digestion is cloned directly to the pGEX-2T vector (Pharmacia). Other bacterial expression vectors may be used. The constructed plasmid is used to transform bacteria *E. coli* strain DH5αF' and this bacterial transformant bearing the recombinant plasmid is used to produce the 28kDa protein using the procedure of Example 1.

* * * * *

Using standard recombinant techniques, a chemically synthesized gene encoding the 28kDa protein may be prepared. The chemically synthesized gene comprises a chemically

synthesized polynucleotide which codes on expression for the amino acid sequence of the 28kDa protein given above.

A rat protein has also been found which binds to LRP and inhibits binding of t-PA to the LRP hepatic receptor. This rat protein has the following amino acid sequence set forth in the Sequence Listing as SEQ ID NO: 5:

```
1                   10                  20                  30
P R L E K L W H K A K T S G S V R L T S C A R V L H K E K I H E Y

                40                  50                  60
N V L L D T L S R A E E G Y E N L L S P S D M T H I K S D T L A S

        70                  80                  90
K H S E L K D R L R S I N Q G L D R L R K V S H Q L R P A T E F E

  100                 110                 120                 130
E P R V I D L W D L A Q S A N F T E K E L E S F R E E L K H F E A

                140                 150                 160
K I E K H N H Y Q K Q L E I S H Q K L K H V E S I G D P E H I S R

            170                 180                 190
N K E K Y V L L E E K T K E L G Y K V K K H L Q D L S S R V S R A

      200
R H N E L
```

Using standard recombinant techniques, a chemically synthesized gene encoding this rat protein may be prepared. The chemically synthesized gene comprises a chemically synthesized polynucleotide which codes on expression for the amino acid sequence of the rat protein given above.

A method of thrombolysis in a mammal is also provided. In accordance with the invention, the method utilizes the t-PA hepatic clearance-inhibiting effect of the 39kDa protein and fragments thereof. The method comprises administering to the mammal, preferably intravenously, a thrombolytically effective amount of t-PA and a t-PA-hepatic clearance-inhibiting effective amount of the 39kDa protein or a t-PA-hepatic clearance-

inhibiting fragment thereof. The preferred amount of t-PA is between 0.15 and 1.5 mg/kg of body weight/dose. The preferred amount of 39kDa protein or fragment administered to the mammal is between 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the preferred amount administered to the mammal is between 38 to 3,800 mg/kg of body weight/dose. The 39kDa protein or fragment thereof may be administered to the mammal up to 20 minutes prior to administering the t-PA.

A composition for treating thrombolytic diseases which may be employed in the method of thrombolysis includes an effective amount of t-PA and an effective amount of a t-PA hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof. The t-PA is preferably present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose. The 39kDa protein or fragment thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the dosage amount is from about 38 to 3,800 mg/kg of body weight/dose.

t-PA is primarily used in the treatment of thrombolytic diseases, but t-PA has also been used for treating myocardial infarctions, tumors via fibrinolysis, and sickle cell anemia via fibrinolysis. The present invention accordingly provides a pharmaceutical composition for a mammalian patient containing t-PA, the improvement comprising further including a t-PA hepatic clearance-inhibiting amount of 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof. The t-PA is

preferably present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose. The 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the dosage amount is from about 38 to 3,800 mg/kg of body weight/dose.

The ability of the 39kDa protein to bind to the t-PA hepatic receptor is demonstrated in the following examples.

EXAMPLE 3

Specific Binding of the 39kDa Protein to the MH_1C_1 Cell surfac

Rat MH_1C_1 hepatoma cells are known to specifically bind ^{125}I-t-PA with high affinity to the cell surface (K_d = 4.9 ± 1.3 nM (SD); Bmax = 78,000 ± 35,000 (SD)) (Bu, G. et al., J. Biol. Chem. 267, 15595-15602). A specific interaction between the 39kDa protein and rat MH_1C_1 hepatoma cells was demonstrated by the following saturation binding analyses.

Cell Culture and Media

Rat MH_1C_1 hepatoma cells were grown in Eagle's minimum essential media (MEM) with Earle's salts (Gibco BRL) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in humidified air containing 5% CO_2 as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. Cell monolayers were used at approximately 90% confluence, and the media was replaced the day prior to each experiment.

Radiolabelling of purified proteins

Recombinant 39kDa protein and t-PA were radiolabelled with carrier-free sodium ^{125}I-iodide (DuPont New England Nuclear

Products) using the Iodogen procedure described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. Specific radioactivities for each radiolabelled protein were between 1 and 2 x 10^7 cpm/μg of protein as measured by γ scintillation spectrometry.

Saturation Binding Analysis

Cells were grown in 12 well dishes to approximately 10^6 cells per well. Monolayers were taken directly from 37°C and cooled on ice. The binding buffer used for the recombinant 39kDa protein was PBSc (phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Each cell monolayer was washed three times with PBSc prior to the addition of binding buffer containing various concentrations of radiolabelled ^{125}I-39kDa protein, either in the presence or absence of an excess of unlabelled protein. Cell monolayers were incubated at 4°C with between 1 and 24 nM recombinant ^{125}I-39kDa protein, either in the presence or absence of 0.5 μM unlabelled protein.

Following a 90 minute incubation, cell monolayers were washed three additional times with PBSc to remove non-specifically associated ligand, and lysed in "low SDS lysis buffer" (0.0625 M Tris-HCl, pH 6.8, with 0.2% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromophenol blue), as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. The amount of cell associated ligand was determined by γ scintillation spectrometry. In cases where t-PA and the 39kDa protein were co-bound, the t-PA binding buffer (PBSa containing 0.2 mM $CaCl_2$ and 10 mM ϵ-amino-n-caproic acid) was used as

described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602.

As shown in FIG. 1A, non-specific binding of ^{125}I-39kDa protein increased linearly over the range of concentrations tested, while total binding increased in a curvilinear fashion, approaching an asymptote above 12 nM. Scatchard analysis (23) (inset to FIG. 1A) is consistent with a single homogeneous population of binding sites and yields an equilibrium dissociation constant (K_d) of 3.2 nM with 220,000 binding sites per cell, for this particular experiment.

The results from ten independent binding experiments indicate a mean K_d value of 3.3 ± 0.9 (SD) nM for ^{125}I-39kDa protein binding to MH_1C_1 cells, with an average of 380,000 ± 190,000 (SD) binding sites per cell, as shown in the following table, Table 1. A represents the mean values with standard deviations for binding of both the 39kDa protein and t-PA to MH_1C_1 cells. The cumulative data from all independent binding experiments was used. B represents the mean values with standard deviation from only simultaneously performed binding experiments. C represents data derived from simultaneous binding experiments performed using either untreated MH_1C_1 cells, or cells pre-incubated for 30 minutes with 0.1% saponin at 4°C.

Tabl 1

Sp cific Binding of ^{125}I-39kDa Pr tein and ^{125}I-t-PA to MH_1C_1 Cells

ligand	preincubation	Bmax ± S.D. (10^3 sites/cell)	Kd ± S.D. (nM)	Experiment (n)
A. Summary				
^{125}I-39kDa	none	380 ± 190	3.3 ± 0.9	10
^{125}I-t-PA	none	78 ± 35	4.9 ± 1.3	7
B. Simultaneous Assays				
^{125}I-39kDa	none	390 ± 150	3.5 ± 0.9	4
^{125}I-t-PA	none	60 ± 35	5.6 ± 1.5	4
C. Simultaneous Assays Performed in the Presence or Absence of Saponin				
^{125}I-39kDa	none	250 ± 8	2.7 ± 1.3	3
^{125}I-t-PA	saponin	515 ± 24	9.1 ± 3.6	3

These data indicate both specific and saturable binding of the 39kDa protein to a single kinetic species of high affinity receptor on the MH_1C_1 cell surface.

The nature of the 39kDa protein binding species was shown by visualizing cell lysates from each binding experiment by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (FIG. 1B). Under the mild conditions used in these experiments (0.2% SDS), the 39kDa protein appeared to bind in isolation, without apparent association with any auxiliary binding proteins. These experiments demonstrate that there is a specific and saturable interaction between the 39kDa protein and an MH_1C_1 cell surface molecule(s).

EXAMPLE 4

Specific Binding of ^{125}I-t-PA and ^{125}I-39kDa Protein t HepG2 Cells

The following saturation binding analyses demonstrated that there is a specific interaction between the 39kDa protein and human hepatoma HepG2 cells. Additionally, the binding parameters to human hepatoma HepG2 cells of ^{125}I-t-PA and ^{125}I-39kDa protein were compared by parallel saturation binding experiments.

Binding studies were carried out at 4°C to avoid possible ligand uptake and degradation. Binding buffer for ^{125}I-t-PA included 10 mM EACA since this lysine analog reduced nonspecific ^{125}I-t-PA binding (presumably to lysine residues on the cell surface) without affecting specific ^{125}I-t-PA binding. (See, Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Cell Culture

Human hepatoma HepG2 cells were maintained in culture as described in (Schwartz, A. L. et al., (1981) J. Biol. Chem. 256, 8878-8881). Cells were cultured in Earle's minimum essential medium (MEM) with glutamine (Gibco Laboratories) supplemented with 10% (v/v) fetal calf serum (Gibco Laboratories), penicillin (100 units/ml), streptomycin (100 μg/ml), and were incubated at 37°C in humidified air containing 5% CO_2. Cultures were supplemented with fresh media

12 hours prior to use. Cell monolayers were generally cultured for two days before use at 80-90% confluence.

Production and Isolation of 39kDa Protein

The 39kDa protein was produced and isolated in accordance with the procedure of Example 1.

Protein Iodination

Iodination of t-PA and 39kDa protein were performed using IODOGEN (Pierce Chemical Co.) as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. The specific activity was generally 5-10 μCi/μg of protein as determined by γ scintillation spectrometry. The unincorporated ^{125}I after gel-filtration purification over a PD-10 column (Pharmacia) was less than 2% of the total radioactivity.

Saturation Binding Assays

Cells were seeded into multiwell (12 wells/plate) disposable plastic tissue culture plates 2 days prior to assay. Ligand binding buffer for t-PA was composed of phosphate-buffered saline (PBS) supplemented with 0.2 mM $CaCl_2$ and 10 mM EACA, whereas PBSc (PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) was used for 39kDa protein binding. Binding experiments were performed at 4°C to prevent possible concomitant internalization during the binding interval. Cell monolayers were washed three times on ice with prechilled binding buffer. Binding was initiated by adding 0.5 ml binding buffer containing selected concentrations of ^{125}I-labelled ligand in the absence or presence of an excess unlabelled ligand (1 μM). After incubation at 4°C for 1.5 hours, buffer containing unbound ligand was removed. Cells

were then washed three times with binding buffer and lysed in 0.0625 M Tris-HCl, pH 6.8, containing 0.2% (w/v) SDS and 10% (v/v) glycerol ("low-SDS lysis buffer"). Radioactivity of cell lysates was quantified by γ scintillation spectrometry. For some experiments, cell lysates were further analyzed by SDS-PAGE and autoradiography as described below. Total binding was determined in the presence of ^{125}I-labelled ligand alone. Nonspecific binding was determined in the presence of excess unlabelled ligand. Specific ligand binding was defined as the difference between total and nonspecific binding.

Specific binding of ^{125}I-t-PA over the concentration range of 0.1-48 nM yielded a curvilinear plot. FIG. 2A shows a representative experiment from seven such experiments performed. Scatchard analysis of the binding data showed a curvilinear plot concave upwards (*inset*, FIG. 2A). This result may be due to either the presence of multiple classes of binding sites with different but fixed affinities, or the existence of site-site interactions of the type defined as "negative cooperativity". (See Meyts, P. D. et al., (1976) J. Biol. Chem. 251, 1877-1888). Due to the type of saturation binding experiments performed, these two possibilities could not be distinguished. When multiple classes of binding sites are assumed, an estimate of two binding sites best fit the data. From the Scatchard analysis shown in FIG. 2A, 40,300 sites are estimated for the high affinity binding (K_d = 1.6 nM) and 146,200 sites (K_d = 32.5 nM) for the low affinity binding. When cell lysat s from selected ligand concentrations were analyzed via SDS-PAGE, specific binding occurred predominantly

in the form of the ^{125}I-t-PA:PAI-1 complex. This is consistent with PAI-1-dependent ^{125}I-t-PA binding on HepG2 cells. (See, Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602).

Specific binding of ^{125}I-39kDa protein to HepG2 cells was performed over the concentration range of 1-24 nM. As shown in FIG. 2B, the 39kDa protein specifically bound to HepG2 cells with a low level of non-specific binding. Saturation of specific binding was observed at ^{125}I-39kDa protein concentrations in excess of 12 nM. Scatchard analysis of the binding data from five such experiments yielded 197,000 ± 23,000 (S.D.) homogeneous high affinity surface binding sites per cell with an apparent K_d = 5.1 ± 0.8 nM (S.D.) (166,000 binding sites per cell and K_d = 4.9 nM for the experiment shown in FIG. 2B). When post-binding cell lysates were analyzed by SDS-PAGE, ^{125}I-39kDa protein was observed at its native molecular mass (i.e., 39kDa) indicating that the binding did not include an SDS-stable complex with other proteins.

* * * * *

The following example demonstrates that low density lipoprotein receptor related protein (LRP) is a receptor for the 39kDa protein.

EXAMPLE 5

Cross-linking of ^{125}I-39kDa Protein to the MH_1C_1 Cell Surface

The 39kDa protein was originally isolated via its ability to co-purify with a low density lipoprotein receptor related protein (LRP) by α2-macroglobulin affinity

chromatography. See Ashcom, J.D. et al., (1990) J. Cell Biol. 110, 1041-1048; Jensen, P.H. et al., (1989) FEBS Lett. 255, 275-280. This low density lipoprotein receptor-related protein (LRP) with a molecular mass of >500 kDa has recently been identified and characterized as a new member of the LDL receptor family (Herz, J. et al., (1988) EMBO J. 7, 4119-4127; Beisiegel, U. et al., (1989) Nature 341, 162-164; Brown, M.S. et al., (1991) Curr. Opin. Lipidol. 2, 65-72). This plasma membrane receptor is unique not only because of its large molecular size but also because of its multifunctional nature in ligand recognition. Studies have shown that LRP mediates binding and endocytosis of both apoprotein E-enriched β-migrating very low density lipoprotein (β-VLDL) (Beisiegel, U. et al., (1989) Nature 341, 162-164; Lund, H. et al., (1989) Proc. Natl. Acad. Sci. USA 86, 9318-9322) and protease- or methylamine-activated α_2-macroglobulin (α_2M) (Strickland, D.K. et al., (1990) J. Biol. Chem. 265, 17401-17404). Based on the following cross-linking studies, it was found that LRP functions as a MH_1C_1 cell specific 39kDa protein receptor.

Metabolic Labelling

Cells were grown in 10 cm dishes as described above in Example 3. Cell monolayers were washed three times with pre-warmed Eagle's minimum essential media (MEM) with Earle's salts lacking L-methionine (Washington University Center for Basic Cancer Research). Following two 15 minute pre-incubations at 37°C with this same media, cells were labelled by the addition of methionine deficient MEM containing 0.4 mCi/ml ^{35}S-methionine (Amersham Corp.).

Labelling was carried out for 4-5 hours at 37°C. After metabolic labelling, cell monolayers were cooled on ice, washed three times with cold PBSc, and incubated with 14 nM of the radiolabelled ^{125}I-recombinant 39kDa protein of Example 3, either in the presence or absence of an excess of unlabelled 39kDa protein, to allow specific binding to the cell surface receptor.

Chemical Cross-Linking

Saturation binding was performed as described above, but with 10 cm dishes of MH_1C_1 cells. Either unlabelled or ^{35}S-methionine labelled cells were used. Following incubation with radiolabelled and unlabelled ligands (39kDa proteins), cell monolayers were washed three times with PBSc to remove non-specifically associated ligand and incubated with either PBSc alone or PBSc containing 0.5-1 mM dithio*bis*(sulfosuccinnimidylpropionate) (DTSSP) (Pierce Chemical Co.), a disulfide bond containing, thiocleavable cross-linker. After one hour, cross-linking reactions were quenched by three washes with tris-buffered saline, and cell monolayers were solubilized in PBSa containing 1% Triton X-100 and 1 mM PMSF. Immunoprecipitations were performed as described below.

Immunoprecipitation

Aliquots of cell lysates from cross-linking and metabolic labelling experiments were added to equal volumes of PBSc containing 1% Triton X-100, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), 0.5% bovine serum albumin , and 1mM PMSF (immunomix). When metabolically labelled cells were

used, lysates were first pre-cleared by an overnight incubation with rabbit pre-immune serum and a subsequent incubation with an excess of protein-A beads (Repligen). Samples were then immunoprecipitated with 0.6 μg of affinity purified α-39kDa antibody or affinity purified rabbit α-human LRP antibody (American Red Cross, Rockville, MD). Affinity purified α-39kDa antibody was prepared in New Zealand white rabbits following injection of purified recombinant 39kDa protein in accordance with the procedure described in Schwartz, A.L. et al., (1983) J. Biol. Chem. 258, 11249-11255.

When unlabelled cells were cross-linked to a single radiolabelled ligand, no pre-immune clearance of cell lysates was necessary, and either α-LRP immune serum or α-39kDa protein immune serum was substituted for the affinity purified antibody. In some cases, samples of cell lysates were also immunoprecipitated with 30 ml of immune α-t-PA serum. Following each immunoprecipitation, bead samples were split in half and boiled in 0.0625 M Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol as described in Laemmli, U.K., (1970) Nature 227, 680-685, either in the presence or absence of 5% 2-mercaptoethanol. Samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with the gels subsequently fixed and vacuum dried. Autoradiography was performed at -70°C with Hyperfilm-MP autoradiography film (Amersham Corp.).

FIG. 3 shows the results from a typical experiment. Under conditions where no cross-linker was used (FIG. 3C and D), a single band corresponding to the 39kDa protein

(arrowhead) was immunoprecipitated only with α-39kDa serum. Samples lectrophoresed under either reducing or non-reducing conditions show that the α-LRP antibody was unable to immunoprecipitate the radiolabelled 39kDa ligand in the absence of cross-linker. Since there was a marked decrease in the amount of ^{125}I-39kDa protein bound in the presence of an excess of unlabelled protein, the association of ligand with its cell surface receptor was shown to be specific. In FIG. 3A and 3B, the radiolabelled 39kDa protein was both bound and cross-linked to the MH_1C_1 cell surface. When samples from each immunoprecipitation were analyzed under non-reducing conditions (FIG. 3A), the complex of ligand associated radioactivity was immunoprecipitable with both α-LRP and α-39kDa antibodies. This complex was of very high apparent molecular weight, remaining largely in the stacking gel during electrophoresis (arrows). Again, this interaction was shown to be specific, as the association of ^{125}I-39kDa with this high molecular weight complex was abrogated in the presence of an excess of unlabelled 39kDa protein. When these same samples were electrophoresed in the presence of 5% 2-mercaptoethanol to dissociate the cross-linker (FIG. 3B), a single 39kDa binding species was seen.

The foregoing experiments demonstrate that there is a specific interaction between the 39kDa protein and the MH_1C_1 cell surface which is mediated, at least in part, by an association with LRP.

EXAMPLE 6

Chemical Cross-linking of t-PA:PAI-1 Compl x to LRP on H pG2 C lls

Studies by Orth, K. et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7422-7426, demonstrated via ligand blotting the binding of t-PA:PAI-1 complexes to isolated LRP immobilized on gels. Endocytosis by LRP and subsequent degradation of t-PA:PAI-1 complexes was also observed in simian COS tumor cells, a non-hepatocyte cell line. The following experiment showed that a direct interaction between the t-PA:PAI-1 complex and LRP in human hepatocytes occurs based on chemical cross-linking following ligand binding to human hepatoma HepG2 cells. In accordance with the present invention, it was hypothesized that binding and subsequent cross-linking of the t-PA:PAI-1 complex to LRP on HepG2 cells should yield a trimeric complex, t-PA:PAI-1-LRP. Therefore, if one component in this trimeric complex is radiolabelled, antibodies to the other components should be able to immunoprecipitate the radiolabelled protein. A thio-cleavable, water-soluble, and membrane-impermeant reagent DTSSP was used as the cross-linker, as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602; and Bu, G. et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431. Following ligand binding and cross-linking, cell lysates were used for immunoprecipitation with various antibodies, in order to characterize the nature of the cross-linked protein complexes. Chemical cross-linking experiments were performed with either ^{125}I-t-PA cross-linked to unlabelled HepG2 cells, or unlabelled t-PA cross-linked to ^{35}S-methionine metabolically-labelled HepG2 cells. Throughout

this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Metabolic Labelling

Cells growing in 10 cm dishes at about 80% confluence were incubated for 30 min at 37°C in two changes of Earle's minimum essential medium lacking L-methionine and containing 2 mM L-glutamine (Gibco Laboratories). Metabolic labelling was initiated by the addition of the above medium supplemented with ^{35}S-methionine (400 μCi/ml)(Amersham). Following incubation for 5 hours at 37°C, cell monolayers were washed with binding buffer and were used for ligand binding and chemical cross-linking experiments as described below.

Chemical Cross-linking

Experiments were performed with either ^{125}I-labelled ligand cross-linked to unlabelled cells or unlabelled ligand cross-linked to ^{35}S-methionine metabolically-labelled cells. After ligand binding at 4°C, each cell monolayer was washed three times with PBSc. Chemical cross-linking was performed by incubating the cell monolayer with PBSc containing 0.5 mM dithio*bis*(sulfosuccinnimidylpropionate) (DTSSP) (Pierce Chemical Co.). After 30 min at 4°C, the reaction was quenched by washing the cell monolayer two times with Tris-buffered saline (TBS). Cells were then solubilized in PBSc containing 1% (v/v) Triton X-100 (Sigma Chemical Co.) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.) ("lysis buffer") for 30 min at 4°C with a brief sonication at

low power output and occasional vortexing. These solubilized cell lysates were subsequently used for immunoprecipitation.

Antibodies

All antibodies used in these studies were rabbit polyclonal antibodies. Anti-t-PA, anti-PAI-1, and anti-39kDa protein antibodies were produced using recombinant proteins. Anti-LRP antibody was generated against purified human placental LRP provided by Dudley K. Strickland (American Red Cross, Rockville, Maryland). Total IgG for each antibody was purified using Protein A-agarose. Specific IgG for each antibody was affinity-purified against its corresponding protein coupled to CNBr-activated Sepharose 4B (Pharmacia).

Immunoprecipitation

Each cell lysate from chemical cross-linking experiments was divided into equal portions depending upon the number of antibodies used. Each aliquot of the cell lysate was then brought to 0.5 ml with lysis buffer and was mixed with 0.5 ml of PBSc containing 1% (v/v) Triton X-100 (Sigma Chemical Co.), 0.5% (w/v) sodium deoxycholate (Sigma Chemical Co.), 1% (w/v) SDS, 0.5% (w/v) bovine serum albumin (Sigma Chemical Co.), and 1 mM PMSF (Sigma Chemical Co.) ("immunomix"). SDS was included in the immunomix to reduce non-specific immunoprecipitation. Primary antibody (10 µl normal rabbit serum (Sigma Chemical Co.), or 1 µg affinity-purified IgG) was added, and the samples were rocked overnight at 4°C followed by incubation for 1 hour at room temperature with 50 µl of protein A-agarose (Repligen). Non-bound radioactivity was removed by washing protein A-agarose beads

three times with immunomix and three times with PBSc. The protein A-agarose beads were divided into two equal parts before the final wash for nonreducing and reducing assays. The immunoprecipitated material was then released from the beads by boiling each sample for 5 min in 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, and 10% (v/v) glycerol ("Laemmli sample buffer") (Laemmli, U. K., (1970) Nature 227, 680-685) with or without 5% (v/v) 2-mercaptoethanol, and was analyzed by SDS-PAGE as described below.

SDS-PAGE and Autoradiography

Samples of cell lysates or immunoprecipitations were analyzed by SDS-PAGE using polyacrylamide slab gels as described in Laemmli, U. K., (1970) Nature 227, 680-685, under reducing or non-reducing conditions. The following pre-stained molecular weight standards from Bio-Rad were used: myosin: 205 kDa; β-galactosidase: 117 kDa; bovine serum albumin: 80 kDa; ovalbumin: 50 kDa. Autoradiography of ^{125}I-labelled proteins was performed with dried polyacrylamide gels using Hyperfilm-MP (Amersham). For fluorography of ^{35}S-labelled proteins, gels were impregnated with Amplify (Amersham), dried, and exposed to films. Films were placed at -70°C for various periods of time as specified in each figure prior to developing.

FIG. 4 shows an experiment with ^{125}I-t-PA binding and cross-linking to HepG2 cells. Cell lysates without or with cross-linking were immunoprecipitated with one of the following antibodies: normal rabbit serum, anti-t-PA antibody, anti-PAI-1 antibody, or anti-LRP antibody. The

immunoprecipitated material was then analyzed by SDS-PAGE under nonreducing (FIG. 4A) or reducing (FIG. 4B) conditions. Under nonreducing conditions and without cross-linking, the ^{125}I-t-PA:PAI-1 complex was immunoprecipitated by both anti-t-PA (lane 2) and anti-PAI-1 antibody (lane 3) confirming the PAI-1-dependent ^{125}I-t-PA binding to HepG2 cells with the ^{125}I-t-PA:PAI-1 complex being the vast majority of the bound ligand. Uncomplexed ^{125}I-t-PA was observed with anti-t-PA antibody (lane 2) but not with anti-PAI-1 antibody (lane 3). The appearance of the uncomplexed ^{125}I-t-PA (about 40% of the total cell-associated ligand radioactivity) in post-binding lysates was predominantly due to dissociation of ^{125}I-t-PA and PAI-1 during immunoprecipitation and washing with the immunomix containing 1% SDS and 1% Triton X-100. When the cell lysates were analyzed directly using low-SDS sample buffer without prior immunoprecipitation, more than 90% of the specific binding species was in the form of ^{125}I-t-PA:PAI-1 complex as appears in FIG. 5B (data not shown). In the absence of cross-linking, anti-LRP antibody did not immunoprecipitate any ^{125}I-labelled ligand (lane 4) indicating that this antibody does not have any cross-reactivity with the ^{125}I-t-PA:PAI-1 complex. However, with chemical cross-linking, anti-LRP antibody (lane 8), in addition to anti-t-PA antibody (lane 6) and anti-PAI-1 antibody (lane 7), immunoprecipitated ^{125}I-labelled ligands. Since anti-LRP antibody does not cross-react with the ^{125}I-t-PA:PAI-1 complex (lane 4), this specific immunoprecipitation by anti-LRP antibody must be a result of the ^{125}I-t-PA:PAI-1 complexes cross-linked to LRP. The ligand-

receptor complexes consisting of ^{125}I-t-PA:PAI-1 and LRP appeared as a broad smear migrating on top of a 7% SDS-polyacrylamide gel with a molecular mass estimated to be greater than 600 kDa. However, when the same material was analyzed by SDS-PAGE under reducing conditions, the radioligand ^{125}I-t-PA:PAI-1 complex and ^{125}I-t-PA appeared following reduction of the thiocleavable cross-linker DTSSP (FIG. 4B). Reduction of the cross-linked material caused additional dissociation of the ^{125}I-t-PA:PAI-1 complex resulting in an even greater amount of free ^{125}I-t-PA. Normal rabbit serum showed no specific interaction with any of the cross-linked material (lanes 1 and 5).

To exclude the possibility that chemical cross-linking of ^{125}I-t-PA:PAI-1 to HepG2 cells generated an antigenic epitope for anti-LRP antibody, unlabelled t-PA was cross-linked to ^{35}S-methionine metabolically-labelled HepG2 cells. This approach allowed direct visualization of the binding protein for the t-PA:PAI-1 complex or the 39kDa protein and the determination of its relationship to LRP. Following ^{35}S-methionine metabolic labeling of HepG2 cells, cell monolayers were incubated with binding buffer alone, or binding buffer containing t-PA (15 nM) or the 39kDa protein (15 nM) (FIG. 6). Cell monolayers in the absence of ligand was lysed directly without chemical cross-linking whereas those following ligand binding were subjected to chemical cross-linking with DTSSP. Each of the cell lysate preparations was then immunoprecipitated with one of the following antibodies: normal rabbit serum (lanes 1, 6, 11),

anti-t-PA antibody (lanes 2, 7, 12), anti-PAI-1 antibody (lanes 3, 8, 13), anti-39kDa protein antibody (lanes 4, 9, 14), or anti-LRP antibody (lanes 5, 10, 15). The immunoprecipitated materials were then analyzed by 7% SDS-PAGE under either nonreducing or reducing conditions.

As shown in FIG. 6, anti-PAI-1 antibody immunoprecipitated PAI-1 from all the HepG2 cell lysates consistent with the well recognized production of PAI-1 by HepG2 hepatoma cells. (See, Bugelski, P. J. et al., (1989) Thromb. Res. 53, 287-303; Owensby, D. A. et al., (1991) J. Biol. Chem. 266, 4334-4340; Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602; Sprengers, E. D. et al., (1985) J. Lab Clin. Med. 105, 751-758). When an ^{35}S-methionine-labelled HepG2 monolayer was analyzed without incubation with either t-PA or 39kDa protein, only the anti-LRP antibody was able to immunoprecipitate the two LRP subunits (515kDa and 85kDa) (lanes 5). However, following binding of unlabelled t-PA and chemical cross-linking, the ^{35}S-labelled ligand-receptor complexes were immunoprecipitable not only by anti-LRP antibody (lanes 10) but also by anti-t-PA antibody (lanes 7) and anti-PAI-1 antibody (lanes 8). When these materials were analyzed under reducing conditions, the LRP subunits, as well as the t-PA:PAI-1 complexes were observed. These results are thus consistent with the nature of the high molecular weight receptor-ligand complexes composed of the t-PA:PAI-1 complex (ligand) and LRP (receptor). Radiolabelling of the t-PA:PAI-1 complex was a result of complex formation between unlabell d t-PA and endogenous ^{35}S-methionine-labelled PAI-1.

When the ^{35}S-methionine-labelled HepG2 cell monolayer was exposed to externally added 39kDa protein, the ^{35}S-methionine-labelled ligand-receptor complex appearing on top of the gel was immunoprecipitable not only by anti-LRP antibody (lanes 15), but also by anti-39kDa protein antibody (lanes 14). Analysis of these cross-linked materials under reducing conditions yielded the 515kDa radiolabelled LRP band when immunoprecipitated with anti-39kDa antibody, further demonstrating the receptor protein for 39kDa protein on HepG2 cells is LRP.

EXAMPLE 7

The 39kDa Protein is Cross-linked Predominately to LRP on the MH_1C_1 Cell Surface

It was found that LRP is the predominant 39kDa protein binding species by cross-linking unlabelled 39kDa protein to the surface of radiolabelled MH_1C_1 cells using the procedures of Example 5 with the following modifications. Labelling was carried out at 37°C for 4-5 hours in methionine deficient Earle's MEM media supplemented to 0.4 mCi/ml with ^{35}S-methionine (Amersham Corp.). Following several washes, labelled monolayers were bound with unlabelled 39kDa protein, and either cross-linked by the addition of 0.5 mM DTSSP, or incubated in PBSc alone. Lysis and immunoprecipitation were carried out as described above in Example 5.

FIG. 7 shows the results from a typical experiment. When radiolabelled MH_1C_1 cells were cross-linked to unlabelled 39kDa protein, a complex of very high apparent molecular weight resulted, which was immunoprecipitable with both the α-39kDa and, to a lesser degree, the α-human LRP affinity

purified antibodies (FIG. 7A, lanes 4-6). Antibodies to human LRP were used as no α-rat LRP affinity purified antibody is presently available. This high molecular weight complex was electrophoretically identical to that seen when radiolabelled ^{125}I-39kDa protein was cross-linked to unlabelled MH_1C_1 cells (compare with FIG. 3A, lanes 2 and 3). In addition to the cross-linked complex, uncross-linked endogenously labelled 39kDa protein (lane 5, *arrowhead*), and both the 520kDa (*closed arrow*) and 85kDa (*open arrow*) subunits of the LRP receptor were visible. When these same immunoprecipitations were electrophoresed in the presence of the reducing agent 2-mercaptoethanol, the cross-linker was cleaved, and the cross-linked complex was dissociated into its component peptides (FIG. 7B, lanes 4-6). In both cases, the cross-linked complex was shown to contain almost exclusively the 520kDa subunit, and to a lesser degree, the 85kDa subunit of the LRP receptor. In lanes resulting from immunoprecipitation with the α-human LRP antibody, less of the high molecular weight cross-linked complex was evident. It is believed that this occurs partly because the recognition epitopes for the α-LRP antibody are obscured by binding of the 39kDa protein.

When metabolically labelled cell lysates which have been bound with, but not cross-linked to, unlabelled 39kDa protein were immunoprecipitated with the α-39kDa antibody, only endogenously labelled 39kDa protein was seen (*arrowhead*). No high molecular weight compl x was evident. Also, in the absence of cross-linker, immunoprecipitation of metabolically

labelled cell lysates with α-human LRP antibodies results in only the 520kDa (*closed arrow*) and the 85kDa (*open arrow*) subunits of the LRP receptor. Again, no high molecular weight complex was evident. Samples electrophoresed under both reducing and non-reducing conditions gave similar results.

This example demonstrates that the LRP is the predominant 39kDa protein binding species on the MH_1C_1 cell surface.

* * * * *

Example 8 demonstrates that both the 39kDa protein and t-PA specifically co-bind to a single LRP molecule.

EXAMPLE 8

t-PA and the 39kDa Protein can be Cross-linked to the Same LRP Receptor Complex

0.5nM ^{125}I-39kDa protein and 20nM unlabelled t-PA were co-bound to MH_1C_1 cells both in the presence and absence of an excess of unlabelled 39kDa protein. Following several washes to remove non-specifically associated ligand, cell monolayers were either incubated in 1mM DTSSP or in PBSc alone. Once cross-linked, cell monolayers were washed, lysed and immunoprecipitated as described above.

FIG. 8 shows the results from a typical experiment. When the ^{125}I-39kDa protein and unlabelled t-PA were co-bound to the MH_1C_1 cell surface and incubated in the absence of cross-linker (FIG. 8C and D), the radiolabelled 39kDa protein was immunoprecipited from cell lysates with only the α-39kDa antibody (*arrowhead*). Neither the α-LRP antibody nor the α-t-PA antibody showed any affinity for the radiolabelled 39kDa ligand. Samples electrophoresed under both reducing and

non-reducing conditions gave similar results. As in Example 3, interaction of the 39kDa protein with the MH_1C_1 cell surface was shown to be specific by abrogation with an excess of unlabelled 39kDa protein. When MH_1C_1 cells were pre-bound with ^{125}I-39kDa protein and unlabelled t-PA, and incubated in the presence of cross-linker (FIG. 8A and B), a high molecular weight complex of ligand-associated radioactivity resulted, which was immunoprecipitable with α-39kDa, α-t-PA, and α-LRP antibodies. Each of these interactions was specific, as each was abolished in samples incubated in the presence of an excess of unlabelled 39kDa protein. This protein complex of ^{125}I-39kDa, t-PA, and LRP was of very high apparent molecular weight as before, remaining largely in the stacking gel during electrophoresis (*arrows*). When these same samples were electrophoresed in the presence of 2-mercaptoethanol to cleave the cross-linker, each resulted in a single radiolabelled band corresponding to the exogenously added ^{125}I-39kDa protein (*arrowhead*).

In order to determine if cross-linking of the 39kDa protein to the MH_1C_1 cell surface generates an epitope for recognition by the α-t-PA antibody, similar experiments were performed with ^{125}I-39kDa protein bound and cross-linked to the MH_1C_1 cell surface, but in the absence of exogenously added, unlabelled t-PA (FIG. 8E and F). In this case, the high molecular weight complex of ligand-associated radioactivity was immunoprecipitable only with α-LRP and α-39kDa antibodies, and not with the α-t-PA antibody.

This experiment shows that the simple cross-linking of radiolabelled 39kDa protein to the MH_1C_1 cell surface does not change its specificity for either the α-t-PA or α-39kDa antibodies. A number of additional co-binding and cross-linking experiments, performed using ^{125}I-t-PA and unlabelled 39kDa protein, gave similar results (data not shown). This data taken together with the data presented above leads to the conclusion that the 39kDa protein and t-PA can bind simultaneously to LRP on the MH_1C_1 cell surface.

* * * * *

It was found from the following examples that endocytosis of the 39kDa protein is receptor-mediated like t-PA and that the 39kDa protein modulates the receptor-mediated endocytosis of t-PA.

EXAMPLE 9

Rapid Uptake and Degradation of ^{125}I-39kDa by MH_1C_1 Cells

It is known that MH_1C_1 cells rapidly endocytose and degrade t-PA (Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). The following single cycle uptake and degradation experiments demonstrate that MH_1C_1 cells similarly endocytose the 39kDa protein.

Endocytosis experiments were performed according to Ciechanover et al., (1983) J. Biol. Chem. 258, 9681-9689 and Owensby et al., (1988) J. Biol. Chem. 263, 10587-10594. 6 well dishes containing 10^6 cells/well were preincubated at 4°C with 14 nM ^{125}I-39kDa protein to allow binding. Following three washes with PBSc (4°C) to remove non-specifically associated ligand, each dish was incubated at 37°C, and a pre-warmed

solution of 200 nM unlabelled 39kDa protein was added to initiate internalization. At each time point, over a period of 60 minutes, one of the dishes was cooled directly on ice, and the overlying media was removed. Precipitation of the overlying media was carried out by the addition of bovine serum albumin to 10 mg/ml and trichloroacetic acid (TCA) to 20%. Both the TCA soluble (i.e., extracellular degraded ligand) and TCA insoluble (i.e., extracellular dissociated ligand) fractions were counted by γ scintillation spectrometry.

In addition, cell monolayers were washed three times with PBSc and incubated 30 minutes at 4°C with a solution of PBSc containing 0.25% Pronase (Calbiochem) to digest membrane-associated ligand. Cells were subsequently removed from each well and pelleted by centrifugation at 12,000 *g*. Both the cell pellets and the supernatants following Pronase treatment were counted by γ scintillation spectrometry to determine both cell associated (i.e., protease resistant) and membrane-associated (i.e., protease sensitive) reactivity.

FIG. 9 shows the results from a typical experiment. As expected, all ligand-associated radioactivity was initially located at the cell surface. Within 3 minutes of warm-up, however, 80% of this population became intracellular (protease resistant) or dissociated into the media as TCA insoluble (undegraded ligand) counts. The intracellular levels of ligand reached a peak value of 50% of total at approximately 10 minut s. Concurrently, the TCA soluble radioactivity, representing internalized and subsequently degraded ligand,

appeared in the overlying media initially at 10 minutes, with plateau levels reached at between 45 and 60 minutes. This kinetic pattern of uptake and degradation of a single cohort of pre-bound ^{125}I-39kDa ligand was identical to that observed for ^{125}I-t-PA with these MH_1C_1 cells.

EXAMPLE 10

Inhibition of ^{125}I-t-PA Uptake and Degradation by the 39kDa Protein on HepG2 Cells

The receptor-mediated endocytosis of the t-PA:PAI-1 complex by HepG2 cells has been previously demonstrated. (See, Owensby, D. A. et al., (1988) J. Biol. Chem. 263, 10587-10594; Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Underhill, D. M. et al., (1992) Blood 80, 2746-2754). It was found from the following experiment that the endocytosis and degradation of the t-PA:PAI-1 complex by HepG2 cells were also via LRP and that the 39kDa protein inhibits the endocytosis and degradation of ^{125}I-t-PA.

Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Ligand Uptake and Degradation

HepG2 cell monolayers seeded in 6-well dishes were incubated at 37°C with 3 nM ^{125}I-t-PA in the absence or presence of various competitors at selected concentrations. After selected intervals, binding buffer overlying cell monolayers was collected, cell monolayers were chilled on ice and washed with prechilled binding buffer for three times to prevent further ligand uptake and degradation. Cellular degradation

of radiolabelled ligand was determined by measuring the appearance of TCA-soluble radioactivity in the overlying buffer, whereas cell-associated radioactivity was determined by lysing the cell monolayers in the low-SDS lysis buffer and measuring the total radioactivity in the cell lysates. Cell-mediated degradation of ^{125}I-t-PA was monitored in the absence or presence of an excess of one of the following competitor proteins: t-PA, 39kDa protein, or BSA.

The results shown in FIG. 10A demonstrate that, in the absence of any competitor protein, the degradation of ^{125}I-t-PA (initial concentration=3 nM) increased almost linearly for at least 4 hours. This observation suggests the existence of an intracellular receptor pool and recycling of the t-PA receptors. (See, Owensby, D. A. et al., (1988) J. Biol. Chem. 263, 10587-10594; Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235). Excess unlabelled t-PA (1 μM) reduced the degradation of ^{125}I-t-PA to about 10% which represents the nonspecific degradation of the ligand. When the 39kDa protein was included in the incubation, specific degradation of ^{125}I-t-PA was inhibited by about 75%. This is consistent with the majority of the specific degradation occurring via LRP. This degree of inhibition of ^{125}I-t-PA degradation by the 39kDa protein is similar to the degree of inhibition of ^{125}I-t-PA binding by the 39kDa protein (see FIG. 5). The control protein BSA had no effect on ^{125}I-t-PA degradation.

To confirm that the inhibition of ^{125}I-t-PA degradation by the 39kDa protein occurs at the step of ligand uptake rather than intracellular degradation, the cell-

associated radioactivity was quantitated during the first hour of endocytosis. As shown in FIG. 10B, about 70% of the specific ^{125}I-t-PA uptake was inhibited by the 39kDa protein, whereas the control protein BSA showed no inhibitory effect on this process.

These results clearly demonstrate that LRP serves as the major PAI-1-dependent endocytosis receptor for t-PA on HepG2 cells, and that the 39kDa LRP receptor-associated protein modulates this interaction.

* * * * *

MH_1C_1 cells rapidly catabolize the 39kDa protein as shown in Example 11.

EXAMPLE 11

Rate of 39kDa Protein Uptake by MH_1C_1 Cells

The following experiment used the procedure described in Schwartz, A.L. et al., (1982) J. Biol. Chem. 257, 4230-4237.

Rate of 39kDa Protein Uptake

Dishes containing monolayers of 10^6 MH_1C_1 cells/well were cooled on ice and washed three times with cold PBSc. Ligand uptake was initiated by the addition of prewarmed (37°C) binding buffer containing 28 nM ^{125}I-39kDa protein, either in the presence or absence of an excess of unlabelled 39kDa protein. Each dish was incubated at 37°C for a period of between 0 and 120 minutes. At each time point, one of the dishes was cooled directly on ice, and the overlying media was removed. Cell monolayers were washed three times with PBSc and lysed in low SDS lysis buffer for the determination of

cell associated radioactivity. Each sample of overlying media was precipitated with TCA as described in Example 9, and the TCA soluble fraction was counted by γ scintillation spectrometry.

FIG. 11 shows the results from a typical experiment. The amount of cell-associated ligand increased rapidly, reaching a plateau at approximately 40 minutes after the transition in temperature. The amount of extracellular degraded ligand exhibited a lag of approximately 20 minutes, after which there began a linear increase which lasted the duration of the experiment. The sum of both cell-associated and extracellular degraded ligand also increased linearly with a slope identical to that of extracellular degraded ligand alone. The slopes of each of these parameters was a measure of the rate of ligand uptake and degradation at 37°C and was determined to be 52 fmol/10^6 cells/minute. This experiment demonstrates a pattern of rapid catabolism of the 39kDa protein by MH_1C_1 cells.

* * * * *

The following examples demonstrate that the 39kDa protein inhibits t-PA binding to hepatic receptors.

EXAMPLE 12

Rat 39kDa Protein Can Efficiently Inhibit t-PA Binding to MH_1C_1 Cells

Using the procedure described in Example 3, the ^{125}I-t-PA binding analyses described below showed the kinetics of inhibition of t-PA binding to MH_1C_1 cells by the native rat 39kDa protein. FIG. 12 shows the results of ^{125}I-t-PA binding analyses performed in the presence of varying concentrations

of either unlabelled t-PA or recombinant rat 39kDa protein as competitors. Unlabelled t-PA inhibits binding of ^{125}I-t-PA with an apparent K_i value of 8 nM (FIG. 12B). The 39kDa protein, on the other hand, competed at a much lower concentration, with an apparent K_i value of 0.5 nM (FIG. 12A). This experiment demonstrates that the 39kDa protein is a strong competitor for ^{125}I-t-PA binding to LRP on the MH_1C_1 cell surface.

EXAMPLE 13

Inhibition of ^{125}I-t-PA:PAI-1 Complex Binding to HepG2 Cells by 39kDa Protein and Anti-LRP Antibody

In the following ligand binding competition experiments, it was found that PAI-1-dependent ^{125}I-t-PA binding was also mediated via LRP. Specific binding of 3 nM ^{125}I-t-PA was performed in the absence or presence of each of three competitor proteins: t-PA, 39kDa protein, or bovine serum albumin (BSA). Each competitor protein was included at increasing concentrations up to more than 100-fold molar excess of the radiolabelled ligand. Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Ligand binding competition assays

Binding of the ^{125}I-labelled ligand was performed as described above in Example 4 in the absence or presence of various competitors at selected concentrations. In those experiments in which both t-PA and 39kDa protein were involved, the t-PA binding buffer was used. Nonspecific binding was determined in the presence of 1 μM unlabelled ligand.

As shown in FIG. 5A, unlabelled t-PA competed with ^{125}I-t-PA for binding with an apparent K_i of about 3 nM. The 39kDa protein also inhibited specific ^{125}I-t-PA binding to a maximum of about 80% (FIG. 5A). The control protein BSA had no effect on specific ^{125}I-t-PA binding indicating that inhibition by the 39kDa protein was not due to a generalized effect but was specific. These results with the 39kDa protein suggest that the majority of the PAI-1-dependent ^{125}I-t-PA binding on the HepG2 cells is via LRP. The observation that the 39kDa protein inhibits only 80% of specific ^{125}I-t-PA binding may indicate the presence of a minor population of t-PA binding sites that are not accessible to or inhibited by the 39kDa protein.

To test whether inhibition of ^{125}I-t-PA binding by the 39kDa protein was due to interference with formation of the complex between ^{125}I-t-PA and endogenous PAI-1, post-binding buffer overlying cell monolayers from the binding assays of FIG. 5A (without any competitor protein or from each of those with 500 nM competitor protein) was analyzed by SDS-PAGE (FIG. 5B). The ^{125}I-t-PA:PAI-1 complex can be seen in the absence of excess unlabelled t-PA (lane 1), but not in the presence of excess unlabelled t-PA (lane 2). Inclusion of excess unlabelled 39kDa protein did not interfere with the formation of the ^{125}I-t-PA:PAI-1 complex (lane 3). The ^{125}I-t-PA:PAI-1 complex observed in the presence of the 39kDa protein is greater than that seen with ^{125}I-t-PA alone and presumably reflects the inhibition of complex binding to HepG2 cells by the 39kDa protein. Cell lysates corresponding to the

overlying buffer shown in lane 1-4 were also analyzed via the same SDS polyacylamide gel (FIG. 5B). Binding of the ^{125}I-t-PA:PAI-1 complex to HepG2 cells is demonstrated in the cell lysates without competitor protein (lane 5). When excess unlabelled t-PA was included, however, specific binding of the ^{125}I-t-PA:PAI-1 complex was absent (lane 6). When the cell lysate with 500 nM 39kDa protein was compared to that with ^{125}I-t-PA alone, specific ^{125}I-t-PA:PAI-1 complex binding was reduced by about 80% (lane 7). The control protein BSA neither interfered with the complex formation (lane 4) nor inhibited specific ^{125}I-t-PA:PAI-1 complex binding (lane 8). These results thus demonstrate that the inhibition of ^{125}I-t-PA binding to HepG2 cells by the 39kDa protein occurs at the level of ^{125}I-t-PA:PAI-1 binding to LRP without interference with the initial complex formation.

Modulation of ligand binding to LRP by the 39kDa protein is not due to competition at a single site with various ligands since previous experiments have shown that LRP ligands (e.g., α_2M^*, t-PA) compete only slightly with the 39kDa protein for binding to LRP. (See, Bu, G. et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431). To test whether ^{125}I-39kDa protein binding is affected by t-PA on HepG2 cells, ligand binding competition experiments were performed. Competitor proteins (t-PA, 39kDa protein, or BSA) at various concentrations were included in ^{125}I-39kDa protein binding. As shown in FIG. 13, specific binding of ^{125}I-39kDa protein was reduced only slightly (about 20%) by excess unlabelled t-PA, whereas the binding was completely inhibited by excess

unlabelled 39kDa protein. These results suggest that the 39kDa protein inhibits t-PA:PAI-1 complex binding indirectly perhaps via inducing a conformational change in LRP unfavorable to t-PA:PAI-1 complex binding. Alternatively, t-PA:PAI-1 complex binding sites on HepG2 cells may account for only a small portion of the 39kDa protein binding sites with most of the binding sites distributed over other parts of the LRP molecule not shared with t-PA.

To test directly whether the majority of PAI-1-dependent ^{125}I-t-PA binding sites is on LRP, ligand binding competition experiments were performed in the presence of anti-LRP antibody. This rabbit polyclonal antibody was generated using purified human placental LRP as antigen. The total IgG fraction was purified and used for competition of ^{125}I-t-PA binding (3 nM). Non-immune rabbit IgG was used as a control. As shown in FIG. 14, specific binding of the ^{125}I-t-PA:PAI-1 complex was reduced by up to 75%, while non-immune IgG had little or no effect. The percentage of reduction on specific ^{125}I-t-PA binding by anti-LRP antibody (i.e. approximately 75%) is similar to that seen with the 39kDa protein (see FIG. 5), further supporting the observation that the predominant binding (~70-80%) of the ^{125}I-t-PA:PAI-1 complex on HepG2 cells is mediated by LRP.

* * * * *

In the following example, it was found that the 39kDa protein effectively inhibited hepatic clearance of t-PA in both *in vitro* and *in vivo* experiments.

EXAMPLE 14

Effect of 39kDa Protein on Hepatic Cl arance f t-PA

39kDa Protein Preparation

The 39kDa protein was isolated from 5 liters of *E. coli* containing the recombinant plasmid according to Example 1 and yielded the GST-39kDa protein. Following thrombin cleavage and removal of the GST, the 39kDa protein was approximately 95% pure as determined by SDS-PAGE and Coomassie/silver staining. The few percent of non-39kDa protein contained within these preparations were unabsorbed fragments of GST. In order to provide a homogeneously pure preparation of 39kDa protein, this partially purified mixture was applied to a heparin agarose column (Sigma Chemical Co.). Following extensive washing with 100 mM NaCl to remove contaminating proteins, the 39kDa protein was eluted at 0.4 M NaCl. This preparation resulted in the greater than 99% homogenous preparation of 39kDa protein as determined by SDS-PAGE and Coomassie/silver staining and shown in FIG. 15.

General Procedures

The following procedures were used throughout this example.

1. Cell Culture

Rat hepatoma MH_1C_1 cells were cultured in accordance with the procedure of Example 3.

2. Protein Iodination

t-PA, 39kDa protein, GST, α1-acid glycoprotein (orosomucoid, OR), and the asialo form of α1-acid glycoprotein (ASOR) were iodinated with ^{125}I as described in Example 4. The

specific activity of the resultant labelled proteins was 5-10 μCi/μg of protein.

3. Protein Conc ntration Determination

Protein concentration was determined using the Biorad protein assay with bovine serum albumin as a standard.

Ligand Binding Assays

Bioactivity was determined on this preparation of 39kDa protein by its ability to inhibit ^{125}I-t-PA to MH_1C_1 cells as described in Example 9. Wells containing approximately 10^6 MH_1C_1 cells were washed and preincubated at 4°C with binding buffer containing the indicated concentrations of unlabelled t-PA or unlabelled 39kDa protein in addition to 3 nM ^{125}I-t-PA. Following incubation for 1.5 hours at 4°C, the medium was removed, the cells washed and the specific ^{125}I-t-PA binding was determined based on the radioactivity measured in accordance with the procedure of Example 4. As seen in FIG. 15, this preparation of 39kDa protein is biologically active against the t-PA receptor with 50% inhibition seen at <1 nM.

In vivo Clearance Determinations

In vivo clearance determinations were performed in the following manner on 200 gm female Sprague-Dawley rats obtained from Charles River Breeders and fed standard lab chow ad lib. Following anesthesia with 12 mg Nembutal, the tail vein was catheterized and a 250 μl solution containing the test protein in normal saline was infused over 30 seconds. Repetitive samples were obtained from the distal tail artery into cooled microtubes containing 2 μl heparin (1:1000). Routinely samples were collected at 30 seconds, 1, 2, 4, 6, 8

and 10 minutes. After the 10 minute peripheral sample was collected, a central blood sample was collected by an open cardiac puncture and the visceral organs (liver, kidney, spleen) were rapidly removed to ice. Aliquots of the liver and entire kidney and spleen were counted for radioactivity determination. Heparinized blood samples were centrifuged to separate plasma from blood cells and an aliquot of plasma, generally 25 μl, was spotted onto Whatman 3M paper, dried, precipitated in 10% trichloracetic acid and radioactivity determined in a Packard Gamma Spectrophotometer.

1. Clearance of t-PA

To demonstrate that t-PA is rapidly cleared from the blood plasma following intravenous administration, adult 200 g rats were anesthetized and administered 30 pmol ^{125}I-t-PA via venous injection as described above. Arterial blood samples were collected at the indicated times, separated and plasma radioactivity determined.

As seen in FIG. 16A, following intravenous administration to each of the eight rats of 30 pmol of ^{125}I-t-PA, the plasma ^{125}I-t-PA was rapidly cleared with a $t_{½}$ of approximately one minute. Less than 10% of the ^{125}I-t-PA remained in blood plasma at 10 minutes. Analysis of the liver at 10 minutes following the administration revealed approximately 90% of the initial plasma radioactivity was found in liver.

2. Effect of Excess t-PA on t-PA Clearance

Intravenous administration to three rats of 400-fold molar excess unlabelled t-PA (12 nmol) one minute prior to

administration of the identical dose of 30 pmol ^{125}I-t-PA resulted in a marked reduction in the plasma clearance of ^{125}I-t-PA as seen in FIG. 16A. The plasma half-life was approximately 9-10 minutes and approximately 40% of the ^{125}I-t-PA remained in the blood at 10 minutes. Analysis of the liver at 10 minutes revealed approximately 70% of the initial plasma radioactivity was found in liver.

3. Effect of 39kDa Protein on t-PA Clearance

Intravenous administration to four rats of 250 nmol of unlabelled 39kDa protein (8,000-fold molar excess) one minute prior to administration of the identical 30 pmol ^{125}I-t-PA also resulted in a marked reduction in the plasma clearance of ^{125}I-t-PA as seen in FIG. 16A. Plasma half-life was approximately 6 minutes. Approximately 30% of the ^{125}I-t-PA remained in the blood at 10 minutes. Analysis of the liver at 10 minutes revealed approximately 80% of the initial plasma radioactivity was found in liver.

4. Clearance of 39kDa Protein

A series of controlled clearance experiments were performed to determine the *in vivo* fate of the administered 39kDa protein. Adult 200 g rats were anesthetized and administered 30 pmol ^{125}I-39kDa protein via venous injection. Arterial blood samples were collected at the indicated times, separated, and plasma radioactivity determined as described above. As seen in FIG. 17, following intravenous administration to the rat of 30 pmol of ^{125}I-39kDa protein, the plasma ^{125}I-39kDa protein was rapidly cleared with a $t_{½}$ of approximately one minute.

The following table, Table 2, shows that the analysis of the liver, kidney and spleen at 10 minutes revealed approximately 70% of the initial radioactivity in liver, 7% in kidney and less than 2% in spleen. Following plasma clearance of ^{125}I-39kDa protein in the rat *in vivo* as described in FIG. 17, the liver, kidney and spleen were harvested at 10 minutes and the radioactivity determined. Each figure is the mean ± S.E.M.

TABLE 2

ORGAN DISTRIBUTION OF ^{125}I-39kDa PROTEIN

Preadministration		**^{125}I-39kDa Protein**	**Liver**	**Kidney**	**Spleen**
(dose)		(dose)	(% initial radioactivity)		
a.	none	30 pmol	69 ± 5	7 ± 1	2 ± 0.2
b.	12.5 nmol	30 pmol	72 ± 3	12 ± 1	2 ± 0.2
c.	50 nmol	30 pmol	25 ± 6	13 ± 2	2 ± 0.6
d.	125 nmol	30 pmol	23 ± 5	31 ± 10	2 ± 0.3

The capacity for hepatic clearance of 39kDa protein is large, but can be saturated as seen in FIG. 17 and Table 2. Intravenous administration of increasing doses of unlabelled 39kDa protein results in a decrease of the fraction of the initial dose found in the liver at 10 minutes. Concomitantly there is an increasing fraction of the initial dose found in the kidney, which indicates that maximal hepatic capacity of 39kDa protein clearance can be readily achieved *in vivo*. Therefore multiple dosing schedules and/or continuous infusion of 39kDa protein should markedly reduce hepatic t-PA clearance *in vivo*.

5. Effect of Excess 39kDa Protein on 39kDa Protein Clearance

Intravenous administration of 12.5 nmol of unlabelled 39kDa protein one minute prior to administration of the identical 30 pmol of ^{125}I-39kDa protein resulted in a prolongation of the plasma half-life to approximately three minutes. Analysis of liver, kidney and spleen at 10 minutes revealed approximately 70% in liver, 12% in kidney, and less than 2% in spleen.

Intravenous administration of 50 nmol of unlabelled 39kDa protein one minute prior to the identical 30 pmol of ^{125}I-39kDa protein resulted in a further marked reduction in the plasma clearance of the ^{125}I-39kDa protein as seen in FIG. 17 with a half-life of approximately six minutes. Analysis of the liver, kidney and spleen at 10 minutes revealed approximately 30% liver, 15% in kidney and less than 2% in spleen.

Intravenous administration of 125 nmol of unlabelled 39kDa protein one minute prior to administration of the identical 30 pmol of ^{125}I-39kDa protein resulted in a further marked reduction in the plasma clearance of the ^{125}I-39kDa protein with a plasma half-life of approximately 9 minutes. Analysis of the liver, kidney and spleen revealed approximately 22% in liver, 33% in kidney and less than 2% in spleen.

6. Clearance of Other Proteins

Not all proteins administered to the rat intravenously are rapidly cleared by liver. Adult 200 g rats were anesthetized and administered via venous injection (a) 5

pmol ^{125}I-α1-acid glycoprotein (OR), and (b) 5 pmol ^{125}I-asialo-α1-acid glycoprotein (ASOR). Arterial blood samples were collected at the indicated times, separated, and plasma radioactivity determined as described above. As seen in FIG. 16B, for example, α1-acid glycoprotein was cleared with a half-time of greater than 90 minutes, whereas its asialo-derivative was rapidly cleared with half-time of approximately 0.5 minute (see Schwartz et al., (1984) CRC Crit. Rev. Biochem. 16, 207-233).

EXAMPLE 15

Effect of 39kDa Protein Fragments on Hepatic Clearance of t-PA

The following experiment demonstrates that the entire 39kDa protein molecule is not required for inhibition of t-PA binding to the hepatic t-PA receptor. The 39kDa protein was chemically cleaved and the individual fragments isolated. These were then tested for inhibition of ^{125}I-t-PA binding to receptors on rat hepatocyte cells. Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard.

Purified 39kDa protein was prepared in accordance with the procedure of Example 14. In order to generate isolated fragments of the 39kDa protein, acid hydrolysis was performed under strict conditions. The 39kDa protein in 0.4M NaCl was incubated for 80 hours at room temperature in 70% formic acid. These acid proteolytic conditions provided for the complete cleavage of the intact 39kDa protein at the Asp-Pro bond at amino acid residues 114-115 and the generation of

two large fragments (~20kDa N-terminal fragment and ~28kDa C-terminal fragment).

Following separation on SDS-PAGE, the intact 39kDa protein, the 20kDa N-terminal fragment, and 28kDa C-terminal fragment were electrophorectically transferred to PVDF paper in accordance with the procedure described in Matsudaira, F., A Practical Guide to Protein and Peptide Purification for Microsequences (1989 Academic Press, NY). The appropriate polypeptide bands were visualized by staining representative gel lanes, cut out of the PVDF paper, and eluted by incubation for 1 hour at room temperature in 1% Triton X-100 in 50mm Tris, pH 9. Reanalysis by SDS-PAGE demonstrates single individual bands of the appropriate molecular weights for the intact 39kDa protein, the 20kDa N-terminal fragment, and the 28kDa C-terminal fragment. Sequencing of these individual protein species at the Washington University Microchemical Core Facility confirmed cleavage of the intact 39kDa protein at the Asp_{114}-Pro_{115} bond.

Thereafter the biological activity of the intact 39kDa protein, the 20kDa N-terminal fragment, and the 28kDa C-terminal fragment were tested by quantitating the inhibition of ^{125}I-t-PA binding to rat liver MH_1C_1 cells. Cells were washed and preincubated at 4°C in binding buffer containing 0-16 nM of the intact 39kDa protein (●), the 20kDa N-terminal fragment (■), the 28kDa C-terminal fragment (▲), or PVDF-elution buffer alone (○) as a control. 4 nM ^{125}I-t-PA was added and the incubation continued for 2 hours at 4°C. Following removal and washing of the unbound ^{125}I-t-PA, the amount of

radioactivity bound to the MH_1C_1 cells was determined as described in Example 14.

As shown in FIG. 18, the intact 39kDa protein inhibited ^{125}I-t-PA binding with 50% inhibition at approximately 1 nM and 90% inhibition at approximately 8-10 nM, consistent with previous observations (Bu et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431; Examples 10, 12, 13, and 14 above). The 20kDa N-terminal fragment was essentially without effect (i.e., approximately 10% inhibition at 16 nM; buffer alone exhibited 20-30% inhibition under these identical conditions). In marked contrast the 28kDa C-terminal fragment was an extremely active inhibitor of ^{125}I-t-PA binding with approximately 50% inhibition at 4 nM and 90% inhibition at 16 nM as shown in FIG. 18.

The nearly equipotent activity of the 28kDa C-terminal fragment compared to intact 39kDa protein strongly suggests that a small fragment of the 39kDa protein, which could include a fragment that overlaps the dividing point between the 20kDa N-terminal fragment and 28kDa C-terminal fragment, or a chemically or genetically related/modified form of the molecule, will be recognized by the t-PA hepatic receptor and may serve as a powerful reagent in inhibiting t-PA clearance *in vivo*.

* * * * *

The above description is meant to be illustrative of the present invention, and not limiting thereof. All explanations of the inventors' theory of the invention are for illustrative purposes only.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANTS: Schwartz, Alan L., Bu, Guojun

(ii) TITLE OF INVENTION: Methods And Compositions For Inhibition Of Hepatic Clearance Of Tissue-Type Plasminogen Activator

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Bryan Cave

(B) STREET: 245 Park Avenue

(C) CITY: New York

(D) STATE: New York

(E) COUNTRY: USA

(F) ZIP: 10167-0034

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.50 inch, double density 720 Kb storage

(B) COMPUTER: IBM PC Compatible

(C) OPERATING SYSTEM: DOS Version 3.3

(D) SOFTWARE: Word Perfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: To be assigned

(B) FILING DATE: 17-DEC-1993

(C) CLASSIFICATION: To be assigned

(vii) PRIOR APPLICATION DATA

(A) U.S. APPLICATION NUMBER: 07/992,827

(B) U.S. FILING DATE: 18-DEC-1992

(C) U.S. CLASSIFICATION: Preliminary Class 514

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Barnhard, Elizabeth M.

(B) REGISTRATION NUMBER: 31,088

(C) REFERENCE/DOCKET NUMBER: 00108/064909

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212) 692-1800

(B) TELEFAX: (212) 692-1900

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 323 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg Glu Ser
 1               5                  10                  15
Gly Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu Lys Ala
            20                  25                  30
Gln Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His Ala Asp
        35                  40                  45
Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu Lys Leu
    50                  55                  60
Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn
65                  70                  75                  80
Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala
                85                  90                  95
Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu
            100                 105                 110
Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly
        115                 120                 125
Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His
    130                 135                 140
His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser
145                 150                 155                 160
Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser
                165                 170                 175
Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu
            180                 185                 190
Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser
        195                 200                 205
His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile
    210                 215                 220
Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu
225                 230                 235                 240
Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys
                245                 250                 255
His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg
            260                 265                 270
His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu
        275                 280                 285
Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val
    290                 295                 300
Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His
305                 310                 315                 320
Asn Glu Leu
```

(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 209 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
 1               5                  10                  15
Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
            20                  25                  30
Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
        35                  40                  45
Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
    50                  55                  60
Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
65                  70                  75                  80
Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
                85                  90                  95
Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
            100                 105                 110
Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu Glu Ala
        115                 120                 125
Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn
    130                 135                 140
His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg His Ala
145                 150                 155                 160
Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu Lys His
                165                 170                 175
Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val Lys Lys
            180                 185                 190
His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His Asn Glu
        195                 200                 205
Leu
```

(4) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCGCGTGGAT CCCCCAGGCT GGAAAAGCTG TGG 33

(5) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCAATGAATT CTCAGAGTCG CTCGCCGTCG CCCAC 35

(6) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 203 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
 1               5                  10                  15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
            20                  25                  30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
        35                  40                  45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
    50                  55                  60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65                  70                  75                  80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
                85                  90                  95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
            100                 105                 110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
        115                 120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
    130                 135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                 150                 155                 160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
                165                 170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
            180                 185                 190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
        195                 200
```

CLAIMS

1. A method of inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* in humans comprising administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof.

2. The method of claim 1, wherein the amount of 39kDa protein or fragment thereof ranges from about 60 to 6,000 mg/kg of body weight/dose.

3. The method of claim 1, wherein the fragment of the 39kDa protein is a 28kDa protein and the amount of 28kDa protein ranges from about 38 to 3,800 mg/kg of body weight/dose.

4. The method of claim 1, wherein the 39kDa protein or fragment thereof is administered 0 to 20 minutes prior to administering the t-PA.

5. The method of claim 1, wherein the hepatic-clearance of t-PA is reduced between 20 and 100%.

6. A 28kDa protein characterized by a molecular weight of 28,000 daltons on SDS-PAGE, stability to acid hydrolysis, solubility in 1% Triton X-100, and possessing biological activity against the t-PA hepatic receptor and reducing hepatic clearance of t-PA between 20 and 100%.

7. A 28kDa protein having an amino acid sequence as set forth in SEQ ID NO: 1:

```
Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg Glu Ser
 1               5                  10                  15
Gly Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu Lys Ala
            20                  25                  30
Gln Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His Ala Asp
        35                  40                  45
Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu Lys Leu
    50                  55                  60
```

```
Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn
 65                  70                  75                  80
Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala
                85                  90                  95
Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu
            100                 105                 110
Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly
        115                 120                 125
Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His
    130                 135                 140
His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser
145                 150                 155                 160
Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser
                165                 170                 175
Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu
            180                 185                 190
Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser
        195                 200                 205
His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile
    210                 215                 220
Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu
225                 230                 235                 240
Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys
                245                 250                 255
His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg
            260                 265                 270
His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu
        275                 280                 285
Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val
    290                 295                 300
Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His
305                 310                 315                 320
Asn Glu Leu
```

8. A chemically synthesized gene encoding a 28kDa protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 2:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
 1               5                  10                  15
Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
            20                  25                  30
Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
        35                  40                  45
Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
    50                  55                  60
Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
 65                  70                  75                  80
Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
                85                  90                  95
Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
            100                 105                 110
```

```
Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu Glu Ala
        115                 120                 125
Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn
    130                 135                 140
His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg His Ala
145                 150                 155                 160
Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu Lys His
                165                 170                 175
Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val Lys Lys
            180                 185                 190
His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His Asn Glu
        195                 200                 205
Leu
```

9. A composition for inhibiting the hepatic clearance of tissue-type plasminogen-activator (t-PA) *in vivo* in humans comprising a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance inhibiting fragment thereof.

10. The composition of claim 9, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

11. The composition of claim 9, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

12. In a pharmaceutical composition for a mammalian patient containing tissue-type plasminogen activator (t-PA), the improvement comprising said composition further including a t-PA hepatic clearance-inhibiting amount of 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof.

13. The pharmaceutical composition of claim 12, wherein the t-PA is present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose.

14. The pharmaceutical composition of claim 12, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

15. The pharmaceutical composition of claim 12, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

16. A composition for treating thrombolytic diseases in a mammal comprising an effective amount of tissue-type plasminogen activator (t-PA), and an effective amount of a t-PA hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof.

17. The composition of claim 16, wherein the t-PA is present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose.

18. The composition of claim 16, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

19. The composition of claim 16, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

20. A t-PA-hepatic clearance-inhibiting protein characterized by binding to low density lipoprotein receptor-related protein (LRP) and inhibiting the binding of t-PA to LRP up to about 80%.

21. A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 39kDa protein and fragments

thereof which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

22. A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 28kDa protein of claim 7 which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

23. A rat protein which binds to low density lipoprotein receptor-related protein, having an amino acid sequence as set forth in SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
 1               5                  10                  15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
            20                  25                  30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
        35                  40                  45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
    50                  55                  60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65                  70                  75                  80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
                85                  90                  95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
            100                 105                 110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
        115                 120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
    130                 135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                 150                 155                 160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
                165                 170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
            180                 185                 190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
        195                 200
```

24. A chemically synthesized gene encoding a rat protein which binds to low density lipoprotein receptor-related protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
 1               5                  10                  15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
            20                  25                  30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
        35                  40                  45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
    50                  55                  60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65                  70                  75                  80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
                85                  90                  95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
            100                 105                 110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
        115                 120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
    130                 135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                 150                 155                 160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
                165                 170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
            180                 185                 190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
        195                 200
```

[received by the International Bureau on 22 June 1994 (22.06.94);
original claims 1-21 unchanged; original claims 22-24 amended (2 pages)]

thereof which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

22. A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 28kDa protein of claim 6 and fragments thereof which bind to LRP and reduce hepatic clearance of t-PA between 20 and 100%.

23. A rat 28kDa protein which binds to low density lipoprotein receptor-related protein and inhibits binding of t-PA to low density lipoprotein receptor-related protein, having an amino acid sequence as set forth in SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ile Ser
 1               5                  10                  15
Val Arg Leu Thr Ser Cys Ala Arg Val Leu His Tyr Lys Glu Lys Ile
            20                  25                  30
His Glu Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly
        35                  40                  45
Tyr Glu Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp
    50                  55                  60
Thr Leu Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile
65                  70                  75                  80
Asn Gln Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro
                85                  90                  95
Ala Thr Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala
            100                 105                 110
Gln Ser Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu
        115                 120                 125
Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys
    130                 135                 140
Gln Leu Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly
145                 150                 155                 160
Asp Pro Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu
                165                 170                 175
Glu Lys Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp
            180                 185                 190
Leu Ser Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
        195                 200
```

24. A chemically synthesized gene encoding a rat protein which binds to low density lipoprotein receptor-related protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ile Ser
 1               5                  10                  15
Val Arg Leu Thr Ser Cys Ala Arg Val Leu His Tyr Lys Glu Lys Ile
            20                  25                  30
His Glu Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly
        35                  40                  45
Tyr Glu Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp
    50                  55                  60
Thr Leu Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile
65                  70                  75                  80
Asn Gln Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro
                85                  90                  95
Ala Thr Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala
            100                 105                 110
Gln Ser Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu
        115                 120                 125
Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys
    130                 135                 140
Gln Leu Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly
145                 150                 155                 160
Asp Pro Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu
                165                 170                 175
Glu Lys Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp
            180                 185                 190
Leu Ser Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
        195                 200
```

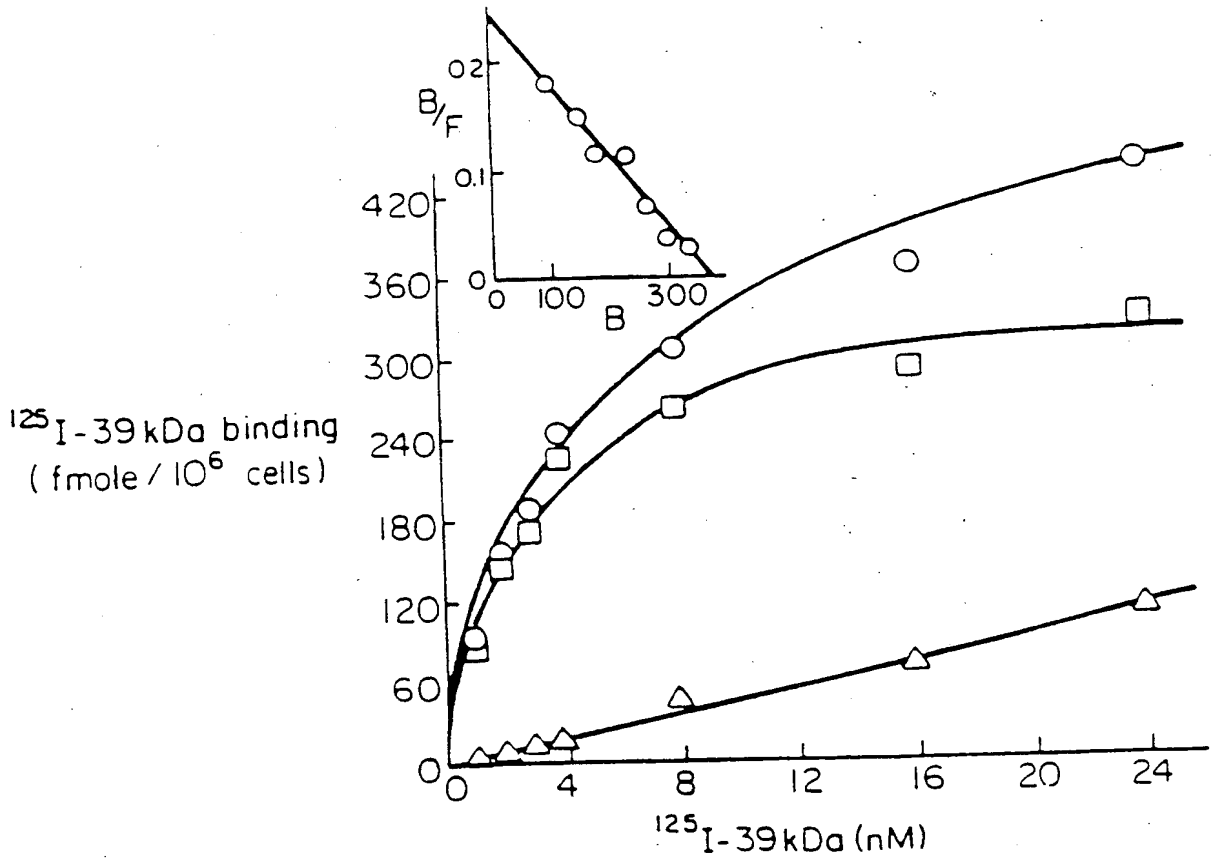


FIG. 1A

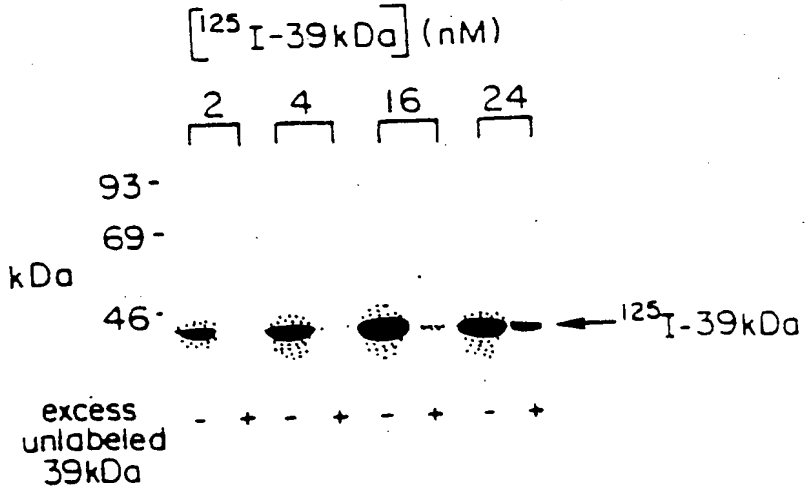


FIG. 1B

RECTIFIED SHEET (RULE 91)